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Contents

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From the Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden

Possible role of thiamine in neuromuscular transmission

By

LENNART WALDENLIND

Received 28 November 1977

Abstract

WALDENLIND, L. *Possible role of thiamine in neuromuscular transmission.* Acta physiol. scand. 1979. *105.* 1–10.

Pyrithiamine (50 mg/kg), a thiamine antagonist, decreased the muscle twitches of the rat masseter muscle at stimulation frequencies above 1 Hz 40–80 min after an i.p. injection. The post-tetanic potentiation (PTP) induced by nerve stimulation of the masseter muscle was abolished by pyrithiamine. Administration of thiamine restored the muscle twitches at stimulation frequencies above 1 Hz and the PTP. The muscle twitches elicited by direct muscle stimulation were not affected by pyrithiamine treatment. The abolishment of the PTP was accompanied by a decrease in thiamine and thiamine-diphosphate. The pyruvate level in the blood was unchanged after pyrithiamine treatment. Oxythiamine, on the other hand, had no effect on the PTP but increased the pyruvate level in the blood. Fern extract, which contains thiaminase I, also abolished the PTP—an effect reversible by the addition of thiamine. The frequency-induced depression of the muscle twitches induced by pyrithiamine was similar to the effect of low doses of d-tubocurarine (8 µg/kg). The results support the hypothesis that thiamine may play a role in neuromuscular transmission.

Thiamine has been described to be of importance for the conduction of nerve impulses (Muralt 1947). It was suggested that thiamine participates in the metabolism of the nerve by acting as a "metabolic" substance and not only as a "catalyst" since more thiamine could be extracted by a Ringer solution from a freeze-dried nerve powder of excited nerves than could be extracted from a corresponding sample of unexcited nerves.

A stimulus-dependent release of thiamine from the curarized phrenic nerve-diaphragm preparation has been described (Waldenlind 1977). To investigate a possible role of thiamine in neuromuscular transmission the effect of thiamine on the miniature end plate potentials of the frog sartorius muscle was studied (Waldenlind *et al.* 1978). Thiamine was found to decrease the miniature end plate potentials significantly and reversibly. Thiamine was also found to bind to isolated nicotinic receptors from *Torpedo marmorata* (Waldenlind 1978) supporting the idea that thiamine binds to nicotinic receptors or to closely related structures. However, the concentration of thiamine used was rather high ($2 \cdot 10^{-4}$ M).

The present study has been made in order to study if thiamine, in addition to its proposed function in neuroconduction, is of importance for neuromuscular transmission. The effect

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Pyrithiamine (30 mg/kg), a thiamine antagonist, decreased the muscle twitches of the rat masseter muscle at stimulation frequencies above 1 Hz 40–80 min after an i.p. injection. The post-tetanic potentiation (PTP) induced by nerve stimulation of the masseter muscle was abolished by pyrithiamine. Administration of thiamine restored the muscle twitches at stimulation frequencies above 1 Hz and the PTP. The muscle twitches elicited by direct muscle stimulation were not affected by pyrithiamine treatment. The abolishment of the PTP was accompanied by a decrease in thiamine and thiamine-diphosphate. The pyruvate level in the blood was unchanged after pyrithiamine treatment. Oxythiamine, on the other hand, had no effect on the PTP but increased the pyruvate level in the blood. Fern extract, which contains thiaminase I, also abolished the PTP—an effect reversible by the addition of thiamine. The frequency-induced depression of the muscle twitches induced by pyrithiamine was similar to the effect of low doses of d-tubocurarine (3 µg/kg). The results support the hypothesis that thiamine may play a role in neuromuscular transmission.

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The present study has been made in order to study if thiamine, in addition to its proposed function in neuroconduction, is of importance for neuromuscular transmission. The effect

of thiamine antagonists (pyrithiamine, oxythiamine, fern extract) on the muscle twitches induced by nerve stimulation has been compared with the effect on muscle twitches induced by direct stimulation.

Methods

Male Sprague Dawley rats (500 g) were anesthetized with pentobarbital (30 mg/kg). The trachea was intubated with a plastic tube just below the larynx. The external jugular vein was cannulated to allow i injections. The blood circulation of the masseter muscle was intact. The masseteric nerve was prepared and cut off proximally Supramaximal stimulation (1.5 times that required for maximal stimulation) of the masseteric nerve was then carried out either directly o by field stimulation (0.2 Hz; 0.2 ms) by a Grass SD 9 stimulator using stainless steel electrodes. The isometric twitches of the masseter muscle were measured by a thread around the teeth connected to a Grass force-displacement transducer (FT 10) nd a Grass poly graph (model 7 B). The contraction force registered is proportional to the tension in the masseter muscle The initial tension was equivalent to 150 g. The contraction force is given in the individual figures. The PTP was provoked by a brief tetanic stimulation at 30 Hz or at 50 H for 5 s. Since the untreated muscle responded with individual twitches t frequencies at 30 Hz (typical for fast muscle) the term PTP is somewhat incorrect. However it is an accepted term for the phenomenon studied and therefore a new term as increase in muscle twitches after stimulation t 30 H was avoided even if this term would hav been more correct.

The PTP was calculated by measuring the increase i height of the fiv first pote tiated muscle twitches. The mean alue for the increase in heights of the five twitches was thereafter expressed as the ratio between the mea increase f r the five muscle tw tches and a nonpotentiated twitch.

All substances were injected i.v in volumes of 0 4 ml (e cept f fern extract where a olume of 2–4 ml was used) All substances were dissolved in 150 mM N Cl and the pH adjusted to 7.3–7 4 with NaOH.

Thiam ne determination

Thiamine and its phosphorylated derivatives were measured in biopsies (~100 mg) from the stimulated masseter muscle The biopsies were homogenized i 3 0.5 ml of 0.1 M HCl, boiled for 30 min in sealed ampoules and then extracted and measured according t Waldenlind (in press). I order to eliminate pyrithiamine fluorescence for the calculations, on aliquot of the sample was treated with 15% KOH for 60 min before ddition of the ferricyanide to destroy the thiamine The thiamine content could then be measured by comparing th fluorescence in the samples which were treated with KOH with those that were not.

Materials

Caffeic acid, d t bocurarine oxythiamine pyrithiamine and thiamine were btained from Sigma Chemical Co

Results

Effects of pyrithiamine on the PTP

Pyrithiamine is a thiamine antagonist that on administration to animals induces the same neurological symptoms as thiamine deficiency (Steyn-Parvé 1967). It has little effect on the coenzyme functions of thiamine diphosphate (Th–DP) (Steyn-Parvé 1967)

Administration of pyrithiamine (50 mg/kg) had no immediate effect on the PTP After 40–80 min, however the PTP disappeared whereas the muscle twitches elicited by low frequency stimulation were unchanged (Fig. 1) (Table 1). No difference was seen if the tetanic stimulation was carried out at 30 Hz for 5 s or at 50 Hz for 20 s. It can also be seen in Fig. 1 that the muscle twitches during the high frequency stimulation were depressed. Direct stimulation of the muscle fibres, however gave rise to PTP (Fig. 1) (n = 3) Thus the effect of pyrithiamine is on neurotransmission and not on the muscle itself Addition of

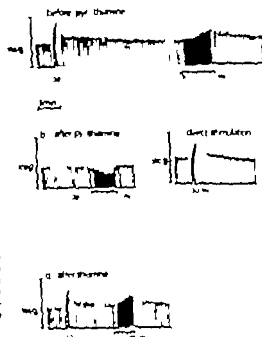


Fig. 1 Effect of pyrithiamine on PTP and muscle twitches at high frequency stimulation. The single muscle twitches were evoked by stimulating at 0.2 Hz and the PTP was evoked by stimulating at 30 Hz for 5 s. The staircase effect was evoked by continuous stimulation at 0.5, 1.0, 2.0, 4.0 and 8.0 Hz respectively. a) before injection of pyrithiamine, b) 35 min after injection of pyrithiamine (50 mg/kg), c) direct muscle stimulation after pyrithiamine administration, the PTP induced by nerve stimulation was abolished, d) 5 min after the injection of thiamine (20 mg/kg) PTP reappeared as did the muscle twitches at high frequency stimulation.

thiamine (10–20 mg/kg) restored the PTP in a few minutes—an effect that lasted at least 60 min. Addition of Th–DP (20 mg/kg) had no restoring effect. In control experiments (n 4) where no thiamine was given, the PTP did not reappear. Instead the ordinary twitches began to decrease gradually 15–25 min after the loss of the PTP. At this stage no recovery of the PTP was achieved with thiamine.

Effect of oxythiamine on the PTP

Oxythiamine is a thiamine antagonist that inhibits the coenzyme reaction of Th–DP (Steyn-Parvé 1967). It is phosphorylated to oxythiamine diphosphate and inhibits thiamine-dependent reactions by competition with Th–DP.

In the present experiments, oxythiamine in doses up to 2 g/kg had no significant effect on the PTP even up to 6 h after administration (Fig. 2) (Table I). The oxythiamine had to be injected in small portions (50 mg/kg). Otherwise a reversible and short-lasting curare-like action was seen.

Effect of fern extract on the PTP

Fern extracts contain factors that inactivate thiamine, one of which has been identified as thiaminase I (Evans 1975). Berüter and Somogyi (1967) have claimed that caffeic acid is the active antithiamine principle.

The fern extract had an effect similar to that of pyrithiamine (Fig. 3) (Table I). The PTP was lost 20–30 min after injection of the extract. If the extract was heated to 100°C for 10 min, 4–7 times larger volumes of the extract had to be injected to get the same effect as that attained with the unheated extract (n 3). The active agent was found to be non-dialyzable.

TABLE I The effects of pyrithiamine, oxythiamine fern tract an d-tubocurarine on PTP are shown. The PTP is calculated by measuring the increase in height of the first 5 potentiated muscle twitches whereafter it is expressed as the ratio between the mean increase in the 5 muscle twitches and a supramaximal nonpotentiated single twitch. In the case of d tubocurarine where total abolishment of the PTP always could be obtained by an additional dose if necessary the effect of the dose necessary for a total block of the PTP is compared with the effect on the single muscle twitches. A negati e value on the PTP means that the post-tetanic twitches are smaller than the pretetanic twitches. The single muscle twitches (in the case of d-tubocurarine) are calculated as the ratio between the twitches after administration of curare and before.

Drug	Size of PTP before drug administration	Size of PTP after drug administration	Size of PTP after thiamine administration	Effect on single muscle tw tches
Pyrithiamine	0.39	0.01	0.32	—
(50 mg/kg)	0.24	0.04	0.26	—
N = 6	0.41	−0.04	0.28	—
	0.18	0.09	0.15	—
	0.37	−0.03	0.31	—
	0.26	0 04	0.32	—
Oxythiamine	0.58	0.58	—	—
(2 g/kg)	0 41	0.36	—	—
n = 4	0 21	0.26	—	—
	0 47	0.39	—	—
Fern extract	0.45	−0.06	0.50	—
n = 4	0.28	0 07	0 17	—
	0.58	0.05	0.36	—
	0.34	−0 01	0.15	—
d Tubocurarine	0.36	0.00	—	0.98
(2–6 μg/kg)	0 32	0.01	—	0.92
= 4	0 45	−0.02	—	0.96
	0.22	0.00	—	0.95

I v injection of thiamine (10–20 mg/kg) restored the PTP (Fig. 3). Caffeic acid did not affect the PTP in doses at least up to 4 g/kg.

Effect of d-tubocurarine on the PTP

The effect of d-tubocurarine was investigated in order to compare its effect with the effects of pyrithiamine and fern extract

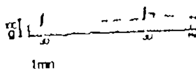
a before



b after oxythiamine



Fig. 2. Effect of oxythiamine PTP) bef re i jection of oxythiamine b) 6 h fter the injection f oxythiamine (2 g/kg). No effect on PTP can be seen.



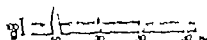




Fig. 3 Effect of fern extract on PTP. Single muscle twitches are evoked by stimulating at 30 Hz for 5 s. a) before injection of fern extract. The PTP was provoked by stimulating at 30 Hz for 5 s. b) 24 min after the injection of fern extract (7.5 ml). PTP has disappeared both after stimulation at 50 Hz for 20 s and at 30 Hz for 5 s. c) 4 min after the administration of thiamine (10 mg/kg) PTP has reappeared. The PTP was provoked by stimulation either at 30 Hz for 5 s or at 50 Hz for 20 s. The contraction force during the 50 Hz stimulation reached the maximum level for the Grass. Therefore the peak tension during these tetani cannot be calculated. In addition, the contractions during the 50 Hz tetani were not isometric since the initial tension was exceeded.

The PTP was inhibited to 50 % by d-tubocurarine (2–6 µg/kg; range of 4 expts.) (Fig. 4). At these doses the ordinary twitches were unaffected. A dose of d-tubocurarine which caused 100 % inhibition of the PTP reduced the ordinary twitches by –8 % only (range of 4 expts.). To inhibit the ordinary twitches by 50 %, doses of 45–75 µg/kg were required (range of 4 expts.). When the muscle twitches were abolished by a high dose of d-tubocurarine (5 mg/kg) PTP still occurred after direct muscle stimulation (Fig. 4) (n = 3).

b. Pyruvate determination

In thiamine deficiency pyruvate levels increase in the blood because of deficient pyruvate decarboxylation (Gubler 1967). The influence of thiamine antagonists on the coenzyme function of Th–DP was therefore followed by measuring the pyruvate levels in the blood.



Fig. 4. Effect of d-tubocurarine on PTP and muscle twitches after high frequency stimulation. Single muscle twitches were evoked by stimulating at 0.2 Hz. PTP was provoked by stimulating at 30 Hz for 5 s and the staircase effect by continuous stimulation at 0.5, 1.0, 2.0, 4.0 and 8.0 Hz (and 16 Hz after curare administration). a) before injection with d-tubocurarine, b) after the injection of d-tubocurarine (8 µg/kg). c) direct stimulation of the anterior muscle after the injection of 5 mg/kg d-tubocurarine. Notice that the magnification in (b) is higher than in (b) and (c).

TABLE II Levels of thiamine and its phosphate derivatives (nmol/g masseter muscle) before and 15, 30, 45 60 and 75 min after the injection of thiamine antagonists. The mean, standard deviation and number of experiments are shown. The biopsies taken at different time intervals are from the same muscle The controls show thiamine and thiamine-phosphate levels in muscles which were stimulated in the same manner as those which received the drugs.

	Before injection	15 min after injection	30 min after injection	45 min after injection	60 min after injection	75 min after injection
Pyrithiamine (50 mg/kg)						
Thiamine	0.29±0.09 n = 14	0.39±0.14 n = 9	0.24±0.11 n = 9	0.17±0.08 n = 11	0.11±0.03 = 7	0.08±0.02 n = 8
Thiamine-phosphates	0.89±0.16 n = 14	0.80±0.15 n = 9	0.78±0.14 n = 9	0.71±0.15 n = 11	0.58±0.37 n = 7	0.55±0.18 = 8
Oxythiamine (1 g/kg)						
Thiamine	0.29±0.09 n = 14		0.27±0.10 n = 5			0.24±0.07 n = 5
Thiamine-phosphates	0.89±0.16 = 14		0.88±0.14 n = 5			0.81±0.17 = 5
Controls, thiamine	0.31±0.14 n = 5	0.28±0.11 n = 5	0.33±0.16 n = 4	0.28±0.13 n = 5	0.34±0.16 n = 5	0.34±0.10 n = 4
Thiamine-phosphates	0.96±0.27 n = 5	0.92±0.22 n = 5	0.87±0.29 n = 4	0.93±0.31 n = 5	0.85±0.27 n = 5	0.93±0.21 n = 4

The mean level of pyruvate in the blood before injecting thiamine antagonists was 169 ± 24 μmol/l (mean ± S D n = 11) Pyrithiamine in doses which abolished the PTP (50 mg/kg) did not significantly change the level of pyruvate 174 ± 29 μmol/l (mean ± S D., n = 8) (measured 80 min after the injection of pyrithiamine) In contrast, the pyruvate level after oxythiamine treatment (2 g/kg) increased to 341 ± 67 μmol/l) (mean ± S D., n = 3) (measured 60 min after the injection of oxythiamine)

Determination of thiamine and its phosphate ester

The levels of thiamine and its phosphate esters in the muscle biopsies before and after injection of thiamine antagonists are shown in Table II Pyrithiamine decreased the levels of thiamine ($p < 0.01$) and of the thiamine phosphates ($p < 0.05$) Oxythiamine had no significant effect.

Discussion

When a skeletal muscle is stimulated at low frequency (0 1–0.2 Hz) with impulses of supra maximal voltage, maximal muscle twitches develop (Brown, Dale and Feldberg 1936). A change in the size of the muscle twitches can be used as an indicator of the efficiency of the neuromuscular transmission (Brown, Dale and Feldberg 1936) If the muscle is stimulated continuously at higher frequencies, then the muscle twitches in fast mammalian muscle will increase (stair-case effect) (Standaert 1964) If the low-frequency induced muscle twitches are interrupted by a short tetanic stimulation, then post-tetanic potentiation (PTP) of the single muscle twitches induced by low-frequency stimulation is seen (Brown and Euler 1938). The PTP is due to an increase in the contractile properties of the muscle fibres (Brown and Euler 1938, Standaert 1964)

The rat masseter muscle used in the present study is a typical "fast" muscle since it responds with individual twitches up to frequencies of 30 Hz. In agreement with the above

mentioned data it was found that the individual muscle twitches increased at high stimulation frequencies, and that PTP developed after moderate tetanic stimulations.

Administration of pyrithiamine or fern extract abolished the PTP after 40–80 min respectively. At the same time a frequency-dependent depression of the muscle twitches was seen (Fig. 1). Thus pyrithiamine caused the fast muscle to behave as a slow muscle. A very similar change was produced by d-tubocurarine (Fig. 4). It has been shown (Buchthal and Lindhard 1937, Engbaek 1948, Naess 1952, Lilleheil and Naess 1961, Bowman and Webb 1973) that the frequency-dependent depression of the neuromuscular transmission is due to a presynaptic effect of d-tubocurarine: the margin of safety for the conduction of trains of nerve impulses in the terminal motor nerve arborization is reduced. Hubbard and Schmidt (1961) and Hubbard *et al.* (1965) showed that d-tubocurarine reduced the magnitude and duration of the negative after-potential in the nerve endings of the phrenic nerve-diaphragm preparation, effects antagonized by neostigmin. These results suggest that the negative after-potential and the presynaptic spike are influenced by acetylcholine. Beranek and Vyskocil (1967) and Hubbard *et al.* (1969) showed that the presynaptic effects by d-tubocurarine were frequency-dependent: the end plate potential was depressed during repetitive stimulation by d-tubocurarine in concentrations that did not abolish depolarization produced by an iontophoretic application of acetylcholine. In spite of the similarities between the effect of pyrithiamine and d-tubocurarine it is improbable that the effects of pyrithiamine described are due to a direct curare-like action of pyrithiamine or the fern extract since curare-like drugs act within a few minutes (Paton and Zaimis 1951). The observation that the effects of pyrithiamine and fern extract were reversed by thiamine are in accordance with the assumption that thiamine is essential for the muscle twitches in the preparation studied. Pyrithiamine did not affect the muscle twitches elicited by direct muscle stimulation. Therefore it is probable that thiamine is important for the neuromuscular transmission.

In an earlier study (Waldenlind *et al.* 1978) it was shown that thiamine in high doses has depressive action on the miniature end plate potentials. Since such an action cannot account for the effects in the present study, a presynaptic effect seems as the most probable explanation for the thiamine effect. However, the specific binding of thiamine to nicotinic receptors (Waldenlind *et al.* 1978) could be in agreement with the view that thiamine binds to "presynaptic nicotinic receptors" and is of importance for their function (nicotinic receptor is defined as a structure which binds d-tubocurarine in low concentrations).

Pyrithiamine has been shown to abolish post-tetanic hyperpolarization (the positive after-potential) in unmyelinated C-fibres of rabbit vagus nerve (Armett and Cooper 1965). Such a change in membrane potential could be due to an impairement of the active transport of sodium and potassium that regenerates and maintains the membrane potential. Barchi (1976) showed that thiamine preserves the electric field across excitable membranes of the node of Ranvier, a process which was strongly temperature dependent thereby suggesting an involvement of a chemical reaction. He also noticed an increase in the refractory period, a widening of the action potentials and depolarizing after-potentials after pyrithiamine treatment, results which could be in accordance with the view that the active transport of sodium and potassium by the Na,K, ATPase across the membrane was disturbed. An impairement of the active transport of ions will lead to difficulties in maintaining the membrane potential

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Controls, thiamine	0.31 ± 0.14 n = 5	0.28 ± 0.11 n = 5	0.33 ± 0.16 n = 4	0.28 ± 0.13 n = 5	0.34 ± 0.16 n = 5	0.34 ± 0.16 n = 4
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and thereby the neurotransmission especially at higher stimulation frequencies. Therefore the present results can be in agreement with the view that thiamine is of importance for the active transport of ions through the nerve terminal membrane.

Since the effects of pyrithiamine and d tubocurarine on PTP in rat masseter muscle were similar it would be of interest if acetylcholine, in addition to its effects on the muscle end plate, has an action on the positive after potential (and thereby on the active transport of sodium and/or potassium) in analogy with pyrithiamine. Acetylcholine has in fact been shown to increase the post-tetanic hyperpolarization (positive after potential) in mammalian nonmyelinated nerve fibres (Armett and Ritchie 1960) The effect of acetylcholine was inhibited by d-tubocurarine (Armett and Ritchie 1960) The positive after-potential is caused by the same mechanism as the post tetanic hyperpolarization which is due to an active transport of potassium into the axons (Greengard and Straub 1958). It is therefore possible that d tubocurarine might affect the degree of polarization of the nerve membrane by interfering with the active transport of sodium and potassium through the nerve membrane a transport which in some way could be under the influence of acetylcholine at high frequency stimulation

Both d-tubocurarine and pyrithiamine inhibit the post-tetanic hyperpolarization of the nerve membrane and might therefore decrease transmitter output during high frequency stimulation. The transmitter output is directly dependent on the degree of polarization of the nerve membrane (Eccles 1964) The assumption that the abolishment of the PTP is due to a block of a presynaptic function of acetylcholine which is related to membrane polarization (Armett and Ritchie 1960, 1961) is in accordance with the results where it was shown that PTP was blocked by d tubocurarine, hexamethonium and AChE-inhibitors (Waldenlind 1978) *In other words the PTP and the membrane polarity after nerve stimulation are* sensitive to the same types of drugs.

The mechanism by which thiamine would maintain and regenerate the membrane potential remains unknown Thiamine could be of importance for acetylcholine synthesis, for generation of *ATP to the ion "pump" it could act* as an ion carrier as *was suggested by Hoffman et al* (1964) or it could be of importance for the function of the presynaptic nicotinic receptors" (Waldenlind 1977) Pyrithiamine is a potent inhibitor of thiamine kinase—an enzyme converting thiamine to Th–DP (Steyn Parvé 1967) Th DP is, according to Koedam (1958), the form in which thiamine is stored in the body He therefore suggested that pyrithiamine interferes with the phosphorylation of thiamine and thereby with the storage of the vitamin in the body In the present study a decrease of both thiamine and Th–DP was noted in the masseter muscle (*Table I*) *Pyrithiamine administration did not elevate the* pyruvate levels in the blood but abolished the PTP Oxythiamine administration, however increased the pyruvate levels of the blood but had no effect on the PTP If the blood levels of pyruvate should reflect the metabolism in the nerve terminals, these results would suggest that the action of thiamine is unrelated to the coenzyme functions of Th–DP However it is uncertain that a lack of effect on the levels of pyruvate in the blood shows that pyruvate dehydrogenase in the nerve terminals is unaffected. Therefore an impairment of the metabolic reactions requiring Th–DP may still be the ultimate mechanism behind the observed effect of pyrithiamine and fern extract on neurotransmission

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From the Departments of Pediatrics, St. Göran Hospital, Karolinska Institute and of Anatomy Karolinska Institute, Stockholm, Sweden and the Department of Cell Biology Institute of Anatomy University of Aarhus, Denmark

Correlation between fluid reabsorption and proximal tubule ultrastructure during development of the rat kidney

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Abstract

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Parallel functional and ultrastructural studies were performed in maturing rats in order to localize factors determining the development of proximal tubular fluid reabsorption. Three groups of hydropenic animals, which were 22 to 24, 28 to 32 and 40 to 45 days old, were studied. Nephron function was evaluated at the single nephron level by micropuncture technique. The ultrastructure of the developing proximal tubules was analysed by morphometric techniques following fixation of single nephrons. Kidney weight proximal convoluted tubule length and diameter increased during postnatal development. SNGFR increased from 2.98 to 8.57 and to 20.5 nl/min in respective group of rats whereas proximal tubular fluid reabsorption Jv (a) increased from 0.15 to 0.22 and 0.34 $\mu m^3 \cdot \mu m^{-2} \cdot s^{-1}$. Parallel to the functional development the relative area of lateral and basal cell membrane increased, resulting in a constant relationship between net fluid reabsorption and the lateral and basal cell membrane area during the fourth postnatal week and then only a slight increase in this relation during the further development. The results suggest that net fluid transport during hydropenia is determined by the amount of available lateral and basal cell membranes where the transporting enzyme for sodium is located.

Key words: Cell membrane and fluid reabsorption, renal structural and functional development, micropuncture of developing tubules, single nephron glomerular filtration during development, Sprague Dawley rats

Clinical studies on the development of renal function have shown that kidneys in preterm infants have a reduced capacity to retain sodium during negative sodium balance as well as to excrete sodium during positive sodium balance (Aperia *et al.* 1972, Aperia *et al.* 1975). Since this homeostatic insufficiency cannot only be explained by a low glomerular filtration rate (Aperia *et al.* 1975), sodium and thereby fluid reabsorption in the immature kidney must during various physiological conditions differ in one or several aspects from that of the mature kidney.

Developmental functional (Horster and Valtin 1970, Spitzer and Brandis 1974) as well as structural (Huber 1905, Larsson 1975) studies on renal tubules are complicated by the fact that at a given age different nephron generations attain different maturational levels. In addition, the characteristic pathway for different transport systems vary in different parts of the nephron. For those reasons it is necessary to restrict each study of mechanisms

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Chemical studies on the development of renal function have shown that kidneys in preterm infants have a reduced capacity to retain sodium during negative sodium balance as well as to excrete sodium during positive sodium balance (Aperia *et al.* 1972, Aperia *et al.* 1975). Since this homeostatic insufficiency cannot only be explained by a low glomerular filtration rate (Aperia *et al.* 1975), sodium and thereby fluid reabsorption in the immature kidney must during various physiological conditions differ in one or several aspects from that of the mature kidney.

Developmental functional (Horster and Valtin 1970, Spitzer and Brandis 1974) as well as structural (Huber 1905, Larsson 1975) studies on renal tubules are complicated by the fact that at a given age different nephron generations attain different maturational levels. In addition, the characteristic pathway for different transport systems vary in different parts of the nephron. For those reasons it is necessary to restrict each study of mechanisms

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radius ( ) the fluid reabsorption per unit luminal surface area as calculated (Györy 1971) as

$$Jv(a) \quad \frac{0.347}{T\,1/2}\ (\mu m^3\ \mu m^{-2}\ sec^{-1})$$

Estimations of the diameter in the oil perfused tubule will be referred to as stop flow determinations.

The reabsorptive rate of the proximal tubule was recorded in eight 22- to 24-day-old rats (group I), in eight 28- to 31-day-old rats (group II) and in five 40- to 45-day-old rats (group III). In the majority of the animals, determinations of total glomerular filtration rate, GFR, and SNGFR, were also made. In 4 animals in group I, in three animals in group II and in two animals in group III, the functional study was followed by fixation of proximal tubule. In addition, proximal tubules were prepared for electron microscopy studies in one 22- to 24-day-old rat, three 28- to 31-day-old rats and two 40- to 45-day-old rats which had not been subjected to previous micropuncture studies. The preparations, including catheterization, infusion and dissection of the left kidney were, however, identical in all rats studied.

Ultrastructural studies. The tubules were fixed *in situ* by microperfusion of a single proximal tubule (Maunsbach 1966) with 3 % glutaraldehyde in 0.1 *M* sodium cacodylate buffer containing 0.5 % lissamine green at pH 7.2. The tubules were located within the same region as those used in the functional experiments and they were perfused with the fixative during 2 min. A small piece of the kidney containing the fixed tubule was excised and fixed for an additional 60 min in the same fixative used for perfusion, post fixed in osmium tetroxide in Veronal acetate buffer at pH 7.2 before dehydration in acetone and embedding in Vestopal W. Ultrathin sections were cut with a LKB Ultratome III, stained with lead citrate and analyzed in JEOL, JEM 100 B or Philips 300 electron microscope.

Electron micrographs at a final magnification of 15 000 were mounted together so that each montage usually covered a whole proximal tubule cross-section. Montages from at least 4 tubules in each age group (see above) were analyzed by morphometric analysis (Weibel *et al.* 1966). A square test lattice was superimposed on the montages and intersections between test lines and lateral and basal cell membranes were counted. The distance between the lines of the lattice corresponded to 0.6 µm on the electron micrograph. The relative area of lateral and basal cell membrane in µm² per µm³ cell volume (surface density) was calculated from the formula given by Weibel *et al.* 1966, $S_v = 2N/L_T$, where N is the number of intersections between the contour of the surface (cell membrane) and test lines, L_T the total length of the test lines expressed in µm. The relative volume of mitochondria was determined by point counting (Weibel *et al.* 1966). The same test lattice was utilized for this purpose and the intersections between the test lines were used as points. The significance of the difference between the means was evaluated by the Student's t-test.

Diameter of proximal tubule lumen. The luminal diameter of the proximal tubule during free flow was determined in three 22- to 24-day-old rats, in three 28- to 31-day-old rats and in three 40- to 45-day-old rats. For this purpose the rats were prepared for micropuncture studies as described above. Photographic recording at 80 times magnification were made 10–15 seconds following the injection of a 10 % lissamine green solution (10 µl/100 g rat) into a femoral artery catheter that had been advanced into the aorta above the entrance of the renal artery. The diameter of randomly selected tubules was measured at 400 times magnification. The diameter of the proximal tubule lumen during stop flow was measured as the width of the oil drop in the light micrographs taken on the tubules whose reabsorptive capacity was determined.

Length of the proximal tubule. Dissection of proximal tubules was carried out in three rats in each age group. For this purpose the left kidney was removed and digested in 20 % hydrochloric acid for 5 h at 37 C. The digested kidney was placed in distilled water. In each kidney four superficial nephrons were dissected and placed intact with the glomerulus and the entire proximal tubule in a drop of distilled water. A camera lucida drawing at 80 times magnification was made of the entire convoluted tubule. The length of the tubule was determined by tracing the drawing with a thread.

Results

The postnatal development of some growth characteristics of the rat kidney during the period between 22–45 days are shown in Table 1. The weight of the kidney increased 2.7 fold from 0.34 g to 0.92 g. The length of the proximal tubule (1st and 2nd segment excluding the 3rd segment, pars recta) increased 1.5-fold, as judged from micro-dissected tubules. The growth in length follows approximately the same pattern as that previously reported in

governing the development of renal sodium absorption to a specific segment of one nephron generation

This report describes the development of the intrinsic reabsorptive capacity of the second segment of the rat proximal tubule (Maunsbach 1966 b) and relates the functional development to the ultrastructural maturation of the proximal tubular cells.

The information obtained will enable a better understanding of the mechanisms governing fluid reabsorption during hydropenia in the maturing rat.

Materials and methods

Experiments were performed in 12 to 45-day-old male Sprague Dawley rats. The rats weighed between 50 and 220 g. They received an ordinary laboratory diet and fluid *ad libitum*. Four hours prior to starting the experiments food and fluid were removed from the cage. The animals were anesthetized with Inactin (Promonta, West Germany) intraperitoneally (80 mg/kg). They were then intubated and placed on a thermostatically controlled micropuncture table. One carotid artery and one jugular vein were cannulated with fine polyethylene catheters. The venous catheter was used for continuous infusion of inulin, Inutest (Laevosan Gesellschaft Linz, Austria) 25 ... normal saline which was given at a rate of 10 µl/g rat/hour. The continuous infusion was preceded by a priming dose of the same solution (10 µl/g rat). The arterial catheter was used for blood sampling and pressure recording. Arterial blood pressure was checked regularly and only those preparations were used in which the pressure was maintained throughout the experimental procedure. A stream of 100 ... oxygen continuously passed in front of the intubation tube. In this way the arterial oxygen tension of the animals was maintained between 85 and 100 mmHg.

The animals were prepared for micropuncture study using the left flank approach. Detailed information of the micropuncture procedure used in this laboratory has been given previously (Aperia and Herin 1975). The left kidney was placed in a lucite cup. A fine polyethylene catheter was inserted into the ureter 5–10 mm below the pelvis. The outer diameter of the catheter ranged between 0.46 and 0.48 mm, whereas the inner diameter of the catheter ranged between 0.40 and 0.42 mm. Single nephron glomerular filtration rate (SNGFR) was determined by spontaneous fluid collection from three to six randomly chosen proximal tubules in each rat. Inulin was determined in tubular fluid samples spectrophotometrically using a micromodification of the anthron method (Hilger et al. 1958). Details of the method used have been described previously (Aperia and Herin 1975). At the time of tubular fluid collection for SNGFR determination, one or two blood samples (20–30 µl each) were taken from the arterial catheter for determination of inulin concentration.

The reabsorptive rate of the proximal tubule was determined with a modification of the Gertz technique (Gertz 1963). In two to six tubules in each rat early parts of the second segment of the proximal tubule, which had a straight appearance were punctured with a single barrelled sharpened glass capillary with a tip diameter on 6–7 µm. The glass capillaries were filled with Sudan stained ricin oil. The approximate location of the puncture site was determined by letting a small oil drop pass through the tubule. An oil drop with the length of at least 4 tubules diameters was then injected into the lumen and was allowed to pass 2.5–4 tubular diameters beyond the capillary tip. Thereafter another oil drop of at least 3 tubular diameters length was injected into the tubular lumen. The fluid between the two oil drops thus consisted of primary urine. The oil drops surrounding the drop of tubular fluid were kept in place or allowed to advance slowly by changing the pressure in the puncturing pipette. When the drops were in the desired position, the diminishing length of the urine drop surrounded by the oil drops was followed by the time sequence photomicrography at 4 sec intervals. For this purpose ... Zeiss Contarex motor driven camera applied to a Zeiss stereomicroscope was used. The lens system permits ... magnification of 80 times. Illumination was provided by a 60 watt/s flash unit synchronized with the camera. The negatives of each exposure were magnified 5 to 7 times. The lengths of the epithelial outlines of each urine drop, i.e. the distance between the bases of the opposing oil menisci was then measured and the mean value for the 2 outline lengths (L) was used. The measurement of L was plotted semilogarithmically against time and the reabsorptive half-time (T1/2) of the droplet could then be determined. The following criteria were used for accepting ... recording: 1) The tubular diameter was constant throughout the procedure indicating a constant intratubular pressure. 2) The initial length of the urine droplet was more than 50 µm. Estimations of linearity were generally confined to the intermediate phase of shrinking. From T1/2 and the tubular

TABLE III Filtration rate and proximal tubule fluid reabsorption in all rats subjected to micropuncture studies.

	Age days	SNGFR nl/min	GFR/g KW ml/min g	Jv ( ) $nm^3 \mu m^{-2} sec^{-1}$
Group I	23	—	—	0.11
	23	1.92	0.39	0.13
	23	—	—	0.13
	23	2.59	0.53	0.16
	23	2.98	0.57	0.17
	23	3.66	0.36	0.16
	24	3.74	0.60	0.19
	[illegible]	2.56	0.49	0.18
Mean value ± S.D.		2.98 ± 0.68	0.49 ± 0.10	0.15 ± 0.03
Group II	28	3.00	0.66	0.23
	28	—	—	0.17
	29	—	—	0.14
	30	6.72	0.71	0.24
	30	11.00	0.79	0.20
	30	7.00	0.73	0.23
	31	11.30	0.84	0.23
	31	9.10	0.80	0.21
	32	—	—	0.27
Mean value ± S.D.		8.37 ± 2.46	0.76 ± 0.07	0.22 ± 0.04
Group III	40	16.3	0.73	0.33
	41	19.7	0.99	0.32
	41	22.1	1.02	0.32
	43	22.0	0.96	0.32
	45	22.2	1.30	0.39
Mean value ± S.D.		20.5 ± 2.6	1.01 ± 0.19	0.34 ± 0.03

The general ultrastructure of tubules prepared for electron microscopy appeared well preserved as shown in Fig. 2. The tubules examined with the electron microscope were almost circular in cross section and were surrounded by a complete basement membrane of about 0.6 µm in thickness in the most mature tubules. The cells at all developmental stages were about 8 µm in height and exhibited a brush border of constant length. The microvilli of the brush border were about 1.2 µm in the 22- to 24-day-old rats and about 1.5 µm in older rats. The increase in the height of the microvilli and the increase in tubular diameter during the development of the proximal tubule resulted in an absolute increase of the apical cell membrane area between groups I and II with a factor 1.5 and between groups II and III with a factor 1.1 per mm tubule.

Mitochondria in the most mature tubules were oriented perpendicular to the basement membrane and in close relation to lateral and basal cell membranes. In the younger tubules, such an obvious orientation of the mitochondria was not present although some of them tended to be oriented in a similar way as in the adult (compare Fig. 3 a and b). The relative volume of the mitochondria (Fig. 4 a) did not change from 23 to 30 days of age. From 30 to 42 days of age the relative mitochondrial volume increased 1.3-fold whereas the absolute volume of the mitochondria increased linearly during the entire development (Fig. 4 b

TABLE I Size of the kidney and the proximal tubule in 22 to 45-day-old rats. Values are mean ±SD

Age (days)	Group I 22 24	Group II 28–32	Group III 40–45
Kidney weight, g	0.34±0 04 (8)[a]	0.53±0.06 (9)[a]	0.92±0.06 (5)[a]
Length of convoluted pro imal tubule mm	4.83±0.32 (12)[b]	5.59±0.55 (12)[b]	7.39±0.66 (12)[b]
Diameter of proximal tubule lumen duri g free flow μm	12.8±1.2 (20)[b]	15 0±1 4 (20)[b]	16.4±1.9 (20)[b]
Diameter of proximal tubule lumen during stop flow μm	25 1±1 1 (8)[c]	27 0±0.7 (9)[c]	29.5±0.9 (5)[c]

[a] Number of rats. Only animals in which functional determinations were made, are included.
[b] Number of tubules measured
[c] Number of rats. Only imals in which functional determinations were made are included. In each rat the mean value of 2–6 observations has been used

Wistar rats (Wahl and Schnermann 1969) The measurement of the luminal diameter of the tubule showed larger values when measured between the oil drops, *i.e* during stop flow condition than during free flow condition This discrepancy has been observed previously (Lewy and Windhager 1968 Nakajima *et al* 1970). During stop flow the oil drop will both flatten the brush-border (Langer *et al* 1968) and distend the tubule which results in an increased diameter of the tubule lumen.

The fractional water excretion (V/GFR) 100 was used as an index of the state of hydration (Table II) (V/GFR) 100 was similar in each group and not significantly different from the finding in 40- to 45-day-old rats that had been fluid deprived for a longer time It was considerably lower than in 40- to 45-day-old rats that had been fluid expanded with isotonic saline amounting to 3 % of the BW

Postnatal development of functional variables is shown in Table III From 23 to 43 days of age, the amount of fluid reabsorbed per μm of apparent proximal tubular lumen per second, Jv (a) increased 2.2 fold as also indicated by the fall in the half time for proximal tubule fluid reabsorption (Fig 1) During the same age interval the filtered load in the superficial nephrons (SNGFR) increased 6.9-fold Total GFR increased from 0.49 to 1 01 *ml/g* kidney weight/min The development of total GFR in relation to kidney weight in the present study continues with approximately the same slope from that reported by Horster and Lewy (1970) from birth to 18-day-old rats. Total GFR per gram kidney weight thus increases rapidly up to about 30 days of age

TABLE II. Fractional water excretion (V/GFR) 100 Values are mean ± S.D Number of animals studied in parentheses.

Group I	0.65±0.18 (5)
Group II	0.79±0.18 (5)
Group III	0 61±0.27 (5)
40-day-old rats following	0 64±0.17 (5)
40-day-old rats following 390 min isotonic volume expansion	7 14±1 64 (5)

Table III. Filtration rate and proximal tubule fluid reabsorption in all rats subjected to micropuncture studies.

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Group I	22	—	—	0.11
	22	1.92	0.59	0.13
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	23	2.98	0.57	0.17
	23	3.66	0.36	0.16
	24	3.74	0.60	0.19
	24	2.96	0.49	0.18
Mean value ± S.D		2.98 ± 0.68	0.49 ± 0.10	0.15 ± 0.03
Group II	28	5.80	0.66	0.23
	28	—	—	0.17
	29	—	—	0.16
	30	6.72	0.71	0.24
	30	11.00	0.79	0.20
	30	7.00	0.78	0.25
	31	11.80	0.84	0.23
	31	9.10	0.80	0.21
	32	—	—	0.27
Mean value ± S.D		8.57 ± 2.46	0.76 ± 0.07	0.22 ± 0.04
Group III	40	16.3	0.78	0.33
	41	19.7	0.99	0.32
	41	22.1	1.02	0.32
	43	22.0	0.96	0.32
	45	22.2	1.36	0.39
Mean value ± S.D.		20.5 ± 2.6	1.01 ± 0.19	0.34 ± 0.03

The general ultrastructure of tubules prepared for electron microscopy appeared well preserved as shown in Fig. 2. The tubules examined with the electron microscope were almost circular in cross section and were surrounded by a complete basement membrane of about 0.6 μm in thickness in the most mature tubules. The cells at all developmental stages were about 8 μm in height and exhibited a brush border of constant length. The microvilli of the brush border were about 1.2 μm in the 22- to 24-day-old rats and about 1.5 μm in older rats. The increase in the height of the microvilli and the increase in tubular diameter during the development of the proximal tubule resulted in an absolute increase of the apical cell membrane area between groups I and II with a factor 1.5 and between groups II and III with a factor 1.1 per mm tubule.

Mitochondria in the most mature tubules were oriented perpendicular to the basement membrane and in close relation to lateral and basal cell membranes. In the younger tubules, such an obvious orientation of the mitochondria was not present although some of them tended to be oriented in a similar way as in the adult (compare Fig. 3 a and b). The relative volume of the mitochondria (Fig. 4 a) did not change from 23 to 30 days of age. From 30 to 42 days of age the relative mitochondrial volume increased 1.3-fold whereas the absolute volume of the mitochondria increased linearly during the entire development (Fig. 4 b

50 Reabsorptive halftime sec

40

30

20

10

0 20 30 40 Age, days 50

Fig. 1 Pro imal tubular reabsorptive halftime in second during postnatal development of 22 to 45-day-old rats.

The cells of the proximal tubules interdigitated more in mature tubules than in the immature tubules resulting in an increase in the area of the lateral and basal cell membranes per μm cell volume (Fig. 5 a) The total volume of one mm tubule wall including the microvilli *i.e* the total volume of the cells in one mm tubule, was calculated from outer and inner tubular diameters determined at free flow conditions. (This procedure gives a ten percentage overestimate of the cell volume since there are large interspaces between the microvilli of the brush border Since this error is the same at all developmental stages comparison between data from the different developmental stages are still valid.) By multiplying the arrived value on total cell volume with the determined relative areas surface densities of lateral and basal cell membranes (Fig. 5) the total area of lateral and basal cell membranes per mm tubule was obtained in each developmental stage This showed an increase in the total area of lateral and basal cell membranes by a factor 1.8 from the youngest to the most mature tubules (Fig. 5 b).

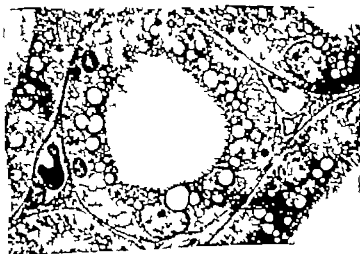


Fig 2. Ultrastructural appearance f a proximal tubule from a 22 day-old rat perfused by microperfusion w th glutaraldehyde. Note the even appearance of the microvill f the brush border and the w ll preserved general ultrastructure of the cells 2 000.



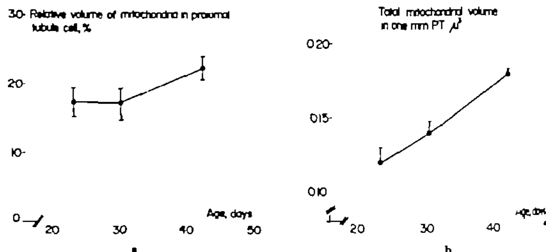


b

Fig. 3 a. Basal part of proximal tubule cell from 22-day-old rat with relatively few mitochondria which have no obvious orientation. Note the few interdigitations of the cell membrane (CM). Fig. 3 b shows an area corresponding to that seen in Fig. 3 a from 43-day-old rat with many mitochondria (M) which are perpendicularly oriented to the basement membrane (BM). Note the numerous interdigitations of the cell membranes (CM). Both Figs. 3 and 3 b are of the same magnification. 30 000.

Fluid reabsorption per unit apical area (excluding the area of the microvilli), Jv (a), increased 2.2-fold. The apparent luminal area (excluding the area of the microvilli) in one mm tubule was obtained from the determination of the inner tubular diameter during free flow conditions. The relation between this area and the total area of the lateral and basal cell membranes in one mm tubule (calculated as above) was determined and used when calculating Jv (B + L) from Jv (a). Jv (B + L) increased 1.5-fold from 23- to 42-day-old rats (Fig. 6). It should be noted that the most pronounced increase in Jv (B + L) will occur between 30 and 42 days of age (1.3-fold, $p < 0.01$) while Jv (B + L) does not change significantly from 23 to 30 days of age.

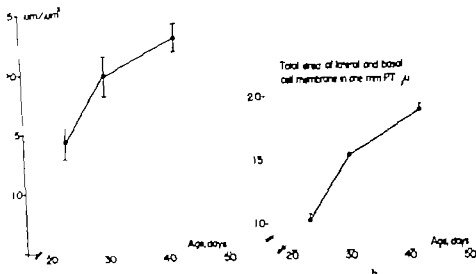
The individual relationship between water flux and basolateral surface membrane in those animals subjected both to functional and structural studies is shown in Fig. 7. The correlation coefficient r = 0.83 is significant.



Figs. 4 a and 4 b Changes of mitochondrial volumes during the postnatal development of the proximal tubule Fig. 4 a shows the relative mitochondrial volume and Fig. 4 b shows the total mitochondrial volume in one mm proximal tubule Vertical bars indicate the standard error The difference between groups II and III in Fig. 4 a is statistically significant ($p < 0.005$).

Discussion

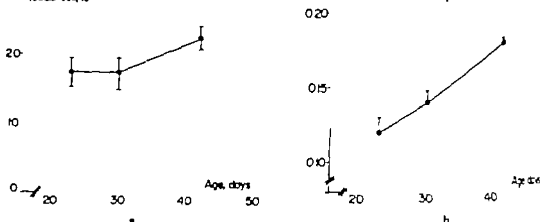
The present study demonstrates a constant relationship between an increasing fluid reabsorption and area of lateral and basal cell membranes during the fourth postnatal week and then only a small increase in this relation during the further studied postnatal development of superficial proximal tubules. It is unlikely that the significant correlation between water flux and basolateral membrane surface is a chance occurrence, since a similar observation has been observed in the developing rabbit proximal tubule where fluid reabsorption has been determined *in vitro* where the tubules were isolated and perfused (Larsson and Horster 1976) It can therefore be suggested that fluid reabsorption in the developing proximal tubules at least during the studied condition of hydropenia may be dependent on the amount of available lateral and basal cell membranes. Most investigators seem to agree that fluid transport in adult proximal tubules will occur both across the epithelial cells (cellular pathway) and the intercellular junctions (paracellular pathway) and will be determined by the osmotic and hydrostatic pressure gradients between the tubular lumen and the periluminal spaces, including the intercellular spaces (Windhager and Giebisch 1976) Water will primarily be reabsorbed because of the osmotic gradient between the lumen and the intercellular spaces (Diamond 1969) created mainly by active transport of sodium to the intercellular spaces (Bank 1976 Green and Giebisch 1975), at least during hydropenia. The sodium transport is considered to be mediated by (Na^{+} + K^{+})-ATPase (Skou 1965) which is located in the cell membrane (Kyte 1976, Sackin and Boulpaep 1975). The present observation of the parallel increase of fluid reabsorption and amount of lateral and basal cell membranes therefore indicates that the amount of sodium transporting enzyme is important for the increased fluid reabsorption. Since (Na^{+} + K^{+})-ATPase was not determined biochemically unequivocal evidence for this hypothesis is not yet available for developing rats However in keeping with this suggestion is result from recent studies in maturing rabbit proximal tubules analysed after isolation and perfusion *in vitro* (Horster



Figs. 5 and 5 b Area of lateral and basal cell membranes of the proximal tubule wall during postnatal development. Fig 5 show the area of lateral and basal cell membranes related to the cell volume (surface density) of the proximal tubule wall and Fig. 5 b show the total area of lateral and basal cell membranes in one mm proximal tubule. Vertical bars indicate the standard error. The difference between groups I and II in Fig. 5 as well the difference between groups I and II and groups II and III in Fig. 5 b are statistically significant (p 0 05).

et al. 1976, Larsson and Horster 1976). These studies showed similar ultrastructural and transporting characteristics of the proximal tubules as did the tubules in the present study (Larsson and Horster 1976). Furthermore, the specific activity of (Na + K)-ATPase was fairly constant during the development (Horster *et al.* 1976) resulting in an increased amount of enzyme since the area of basal and lateral cell membranes increased. The parallel developmental pattern for the relative mitochondrial volume and fluid reabsorption related to the sum of the lateral and basal cell membrane may reflect an increased energy requirement for the active sodium transport.

Previous studies performed on the *in vitro* perfused isolated rabbit proximal tubule have shown that the influence of transepithelial hydrostatic and oncotic pressure gradients on net volume flow is significantly higher in proximal tubules from 2 to 6-day-old rabbits than in proximal tubules from adult rabbits (Horster and Larsson 1976). Simultaneously performed ultrastructural studies using electron dense tracers showed that the proximal tubular epithelium from the postnatal rbabits is more leaky than that from adult rabbits. This indicates that the change in hydraulic conductance is due to alteration of the paracellular pathway It seems very likely that this maturational change will also influence the development of transepithelial fluid transport in the rat proximal tubule. The resistance of the paracellular shunt will, however vary with the state of hydration and will be



Figs. 4 a and 4 b. Changes of mitochondrial vol mes duri g the postnatal development of the proxim tubul Fig 4 a shows the relative mitochondrial volume and Fig. 4 b shows the total mitochondrial volume in one mm proximal tubule Vertical bars indicate the standard error The difference between groups II nd III i F g. 4 is st tistically significant ($p < 0.005$).

Discussion

The present study demonstrates a constant relationship between an increasing fluid reabsorption and area of lateral and basal cell membranes during the fourth postnatal week and then only a small increase in this relation during the further studied postnatal development of superficial proximal tubules. It is unlikely that the significant correlation between water flux and basolateral membrane surface is a chance occurrence, since a similar observation has been observed in the developing rabbit proximal tubule where fluid reabsorption has been determined *in vitro* where the tubules were isolated and perfused (Larsson and Horster 1976) It can therefore be suggested that fluid reabsorption in the developing proximal tubules at least during the studied condition of hydropenia may be dependent on the amount of available lateral and basal cell membranes. Most investigators seem to agree that fluid transport in adult proximal tubules will occur both across the epithelial cells (cellular pathway) and the intercellular junctions (paracellular pathway) and will be determined by the osmotic and hydrostatic pressure gradients between the tubular lumen and the periluminal spaces, including the intercellular spaces (Windhager and Giebisch 1976). Water will primarily be reabsorbed because of the osmotic gradient between the lumen and the intercellular spaces (Diamond 1969) created mainly by active transport of sodium to the intercellular spaces (Bank 1976, Green and Giebisch 1975), at least during hydropenia. The sodium transport is considered to be mediated by (Na + K)-ATPase (Skou 1965) which is located in the cell membrane (Kyte 1976, Sackin and Boulpaep 1975) The present observation of the parallel increase of fluid reabsorption and amount of lateral and basal cell membranes therefore indicates that the amount of sodium transporting enzyme is important for the increased fluid reabsorption. Since (Na + K)-ATPase was not determined biochemically unequivocal evidence for this hypothesis is not yet available for developing rats. However in keeping with this suggestion is result from recent studies in maturing rabbit proximal tubules analysed after isolation and perfusion *in vitro* (Horster



Fig. 7. Correlation between proximal tubular fluid reabsorption related to the luminal area (J (a)) and the area of basal and lateral cell membranes related to the luminal area (excluding the area of the microvilli) during the postnatal development of the proximal tubule. Correlation coefficient 0.13.

studied the scatter of the values for tubular reabsorptive capacity was fairly small. This suggests that the reproducibility of the method used is satisfactory when applied to a homogenous group of rats.

The question whether the method will allow comparison of results obtained from rats of different ages must, however, be considered. The reabsorptive rate of the tubules determined with shrinking drop technique has been found to be influenced by the geometry of the tubule (Györy 1971) and the intraluminal hydrostatic pressure (Grandchamp and Boulpaep 1972). The influence of the first factor will to a large extent be overcome if the reabsorptive rate is expressed per unit apparent tubular lumen area (Györy 1971). Since this was done in this study the change in tubular diameter that occurred during development still probably does not invalidate comparison between the groups.

The intraluminal hydrostatic pressure was not recorded. In order to make the experimentally induced pressure changes comparable among the different groups, however, the Gertz technique (Gertz 1973) was modified so that the tubule was only punctured once and the tips of the puncturing pipette were adapted to the size of the tubule. The fact that the quotient between the free flow and stop flow diameter was almost constant in the different age groups studied also suggest that the effect on intraluminal pressure of puncturing the tubule and injecting oil was comparable in all rats studied (Table II).

Part of this study was presented at the VIth International Congress of Nephrology in Florence, Italy 1975 (Abstract no 80). This study was supported by the Swedish Research Council Proj no B76-19X-03644-03B and grant from the Karolinska Institute (Reservationsanslaget). We thank Mrs Ann-Christine Eklöf and Miss Ann-Christine Ermathing for excellent technical assistance.

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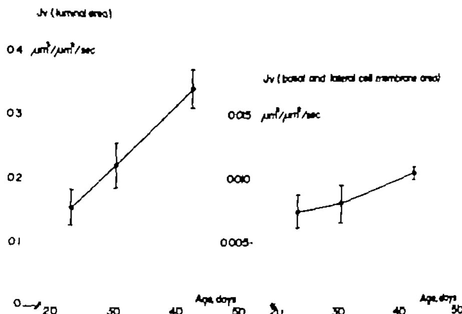


Fig. 6 Proximal tubular fluid reabsorption related t the luminal are (J (a)) (left) and fluid reabsorption to the area of lateral and basal cell membranes during postnatal development (right). Vertical bars indicate the standard error The difference between groups I and II and between groups II and III (left) is statistically significa t ($p < 0.01$) as well as the difference between groups II and III (right) ($p < 0.01$).

most pronounced during hydropenia (Grandchamp and Boulpaep 1974) Thus it has been assumed that during hydropenia the transepithelial sodium transport will even in leaky epithelia be the major determinant of proximal tubular water reabsorption occurring both in the cellular and paracellular pathways (Horster and Larsson 1976) It is therefore suggested that in the hydropenic immature animal, proximal tubular fluid reabsorption is more dependent by the availability of membrane bound active transporting enzyme for sodium than during hydration or extracellular volume expansion.

During the period between 22 and 44 days of rat postnatal development, the proximal tubular reabsorptive rate during hydropenia increased 2.2-fold Assuming a constant diameter of the convoluted proximal tubule, the inner surface area of the total convoluted tubule would be $2\pi rL$ μm where r is the radius and L is the length of the proximal tubule. Using the values from Table I it is found that the surface area will increase 1.9-fold from 22 to 44 days of life. The filtered load in the superficial nephrons increases almost 7 fold, *i.e.* somewhat more than the product of Jv (a) and total lumen area. This indicates either an increased reabsorption per unit filtered load in the proximal tubule of the young rat or a non-homogenous development of the different parts of the proximal tubule

The present study was performed after only four hours of food and fluid deprivation. Pilot studies showed that longer periods of food and fluid deprivation resulted in functionally unstable animals, in particular the younger animals. The low fractional water excretion in all age groups studied, which did not differ significantly from the findings in 40- to 45-day-old rats that were subjected to 12 hours food and fluid deprivation, does, however suggest that the animals were in a satisfactory stage of hydropenia. Within each group

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Ventilatory and occlusion pressure response to CO and hypoxia with resistive loads

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Abstract

Barnett T. B. and B. Rasmussen *Ventilatory and occlusion pressure response to CO and hypoxia with resistive loads.* Acta physiol. scand. 1979. *105* 23-32.

Steady-state responses to hyperoxic hypercapnia and eucapnic hypoxia were measured both as minute ventilation (V) and as inspiratory mouth occlusion pressure (P) with and without 25 cm H_2O/l/s added resistance (R). Reduction in slope of the ventilatory response to CO_2 with R was highly significant in all subjects whereas the response to hypoxia was barely significantly reduced in 1 subject and not significantly decreased in two. Although P was higher with than without R under all conditions, the slope of the P response to CO_2 with R was not increased in two subjects and only slightly increased in the third. The slope of the P response to hypoxia was significantly greater in all subjects with R. Expiratory reserve volume was increased with R but the change was the same with hypoxia and hypercapnia. We conclude that ventilation is better maintained with resistive loading during hypoxia than during hypercapnia and that this results from greater force output of inspiratory muscles as reflected by higher P. This suggests greater neural output to these muscles.

An increase in airway resistance, whether with mechanical resistors (Cherniack and Snidal 1956) or with increased density of respired gases (Doell *et al.* 1973) is associated with a reduction in the ventilatory response to CO. There is less agreement about the effect of increased flow resistance on the ventilatory response to hypoxia. An earlier study in this laboratory (Barnett and Rasmussen 1970) showed that the ventilatory response to eucapnic hypoxia was reduced by adding external airway resistance. It appeared, however, that this effect of increased resistance was less marked than was the effect of the same added resistance on the ventilatory response to CO_2. There have been more recent reports describing a difference in the effect of resistive loading on the ventilatory responses to these two stimuli (Doell *et al.* 1973, Levison and Cherniack 1971). Others have emphasized the similarities between hypoxic and hypercapnic responses during loaded breathing (Rebuck *et al.* 1975, Rebuck and Juniper 1975).

If ventilation is indeed better maintained in the presence of resistive loads when the principal added stimulus is hypoxia then it might be predicted that the inspiratory force

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Ventilatory and occlusion pressure response to CO and hypoxia with resistive loads

By

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Received 10 January 1978

Abstract

BARNETT, T. B. and B. RASMUSSEN. *Ventilatory and occlusion pressure response to CO and hypoxia with resistive loads.* Acta physiol. scand. 1979. *105*. 23–32.

Steady-state responses to hyperoxic hypercapnia and eucapnic hypoxia were measured both as minute ventilation (V) and as inspiratory mouth occlusion pressure (P) with and without 25 cm H_2O l/s added resistance (R). Reduction in slope of the ventilatory response to CO_2 with R was highly significant in all 3 subjects whereas the response to hypoxia was barely significantly reduced in 1 subject and not significantly decreased in two. Although P was higher with than without R under all conditions, the slope of the P response to CO_2 with R was not increased in two subjects and only slightly increased in the third. The slope of the P response to hypoxia was significantly greater in all subjects with R. Expiratory reserve volume was increased with R but the change was the same with hypoxia and hypercapnia. We conclude that ventilation is better maintained with resistive loading during hypoxia than during hypercapnia and that this results from a greater force output of inspiratory muscles as reflected by a higher P. This suggests a greater neural output to these muscles.

An increase in airway resistance, whether with mechanical resistors (Cherniack and Snidal 1956) or with increased density of respired gases (Doell *et al.* 1973) is associated with a reduction in the ventilatory response to CO. There is less agreement about the effect of increased flow resistance on the ventilatory response to hypoxia. An earlier study in this laboratory (Barnett and Rasmussen 1970) showed that the ventilatory response to eucapnic hypoxia was reduced by adding external airway resistance. It appeared, however, that this effect of increased resistance was less marked than was the effect of the same added resistance on the ventilatory response to CO. There have been more recent reports describing a difference in the effect of resistive loading on the ventilatory responses to these two stimuli (Doell *et al.* 1973, Lewison and Cherniack 1971). Others have emphasized the similarities between hypoxic and hypercapnic responses during loaded breathing (Rebuck *et al.* 1975, Rebuck and Juniper 1975).

If ventilation is indeed better maintained in the presence of resistive loads when the principal added stimulus is hypoxia then it might be predicted that the inspiratory force-

output response during resistive loading would be increased more by a hypoxic stimulus than by a hypercapnic stimulus which elicits the same increase in ventilation in the absence of added resistance ("equivalent stimulus") To test this hypothesis the present study was designed to measure and compare the responses to hypoxia and to hypercapnia both in terms of minute ventilation (V_E) and in terms of mouth occlusion pressure, *i.e.* mouth pressure after the first 0 1 s of an occluded inspiration ($P_{0.1}$) This measurement reflects the force of approximately isometric contraction of inspiratory muscles and therefore the neural output to these muscles (Whitelaw *et al* 1975)

Methods

3 healthy young adults well trained as subjects in respiratory experiments were used in this study The two male subjects were 30 (TH) and 26 (HM) years old, the one female (BS) was 25 years old For purposes of obtaining informed consent the experimental conditions were described to all subjects. They were not made aware of the aims of the experiments and were not told which type of experiment was to be done on a given day These steady-state experiments were begun at 9 AM with the subject comfortably seated in a semireclining position They read material of their own choice during the experiments. Hypoxic and hypercapnic gas mixtures were delivered from pre-mixed tanks via two flowraters for adjustments of the final mixture and were humidified before collection in a bag reservoir for delivery to a low resistance two-way valve. Expired air was passed through a dry gasmeter or into Douglas bags for volume measurements and gas analysis.

All experiments were done in the same way and were of 4 types hypoxia without added resistance (R), hypoxia with R CO_2 without R CO_2 with R. Only one type of experiment was done each day and consisted of 3 steady-state periods of 15–20 min separated by 5–10 min rest periods. The following sequence was used in all experiments. atmospheric air first level of test gas, second level of test gas. Ventilation and endtidal CO_2 were continuously monitored. Data points were established from measurements made during intervals of 2 5 min at the end of each steady state period. A total of 49 expts. of all types were performed. In all studies with hypoxia, CO_2 was included in the mixture to maintain a normal end tidal CO_2. Mean end-tidal CO_2 tensions ($\pm$1 S.D.) in the 3 subjects were 39.39 ($\pm$0 48), 39 41 ($\pm$0.96) mmHg and 39 13 ($\pm$0.60) with R and 39 69 ($\pm$0.50), 39 45 ($\pm$0.69) and 39.55 ($\pm$0 57) without R. Experiments with hypercapnia were performed in the same way with inspired CO_2 regulated to obtain steady-state hyperventilation at two levels approximating the two levels obtained with hypoxia. Alveolar O_2 tension was kept between 150 and 200 mmHg during the experiments with hypercapnia.

Resistive loading was with porous discs warmed to prevent condensation and interposed between the subject and the two-way valve thus imposing added resistance during both phases of the respiratory cycle The resistance of the discs was 25 cm H_2O/l/s and linear between 0.2 and 1 0 l/s. In hypoxic experiments with R were the alveolar P_{O_2} and end-tidal CO_2 (see above) adjusted to the same level as in experiments without R. From experience with our previous experiments it became possible to mix gases to obtain the desired ventilation and alveolar gas concentrations. Thereafter the experiments were done in random sequence over a period of approximately 8 months.

Inspired and expired gas samples were analyzed either by the micro Scholander method or with a Beckman Model LB-1 CO_2 analyser and a Servomex oxygen analyser The latter instruments were calibrated with gases of known concentration. End-tidal CO_2 tension ($P_{ET_{CO_2}}$) was calculated from the end of expiratory plateau of the record from Beckman infra red CO_2 analyser Sampling was carried out by micro sampler from the external airway distal to the resistor discs and corrections were made in V_E for gas removed through the pump. The CO_2 analyser was calibrated with gases of known CO_2 concentrations immediately before and immediately after each observation period. Using $P_{ET_{CO_2}}$ in the Bohr formula, as a substitute for P_{aCO_2}, dead space (V_D) was calculated. Assuming the same V_D for O_2 and CO_2 alveolar oxygen tensions ($P_{A_{O_2}}$) were calculated from the Bohr formula. All calibration gas mixtures were analyzed by the micro Scholander method. With simultaneous analysis of arterial and arterialized capillary blood along with end-tidal and alveolar gases, we have shown that the added resistance does not change alveolar-arterial P_{O_2} gradient or end-tidal-arterial P_{CO_2} gradient (Barnett and Rasmussen 1970).



Fig. 1. Ventilatory responses to CO_2 are show in the plots on the left and responses to hypoxia on the right. Open circles represent measurements ithout added resistance, closed circles measurements with added resistance. P_{ETCO_2} levels during hypoxia are given in the text.

For purposes of continuous monitoring of ventilation, expired gas as passed through dry gasometer equipped ith potentiometer. For final measurements of minute ventilation and collection of samples for analysis the expired gas was diverted into Douglas bags. All volumes ere converted to BTPS. Respiratory frequency was obtained from the CO_2 analyser tracing.

Mouth occlusion pressure was measured in most experiments by method described elsewhere (Rasanen and Barnett 1976) in hich manual lever is opened slowly during the expiratory phase allowing compressed air to advance rubber ball into an opening hich occludes the inspiratory airway. Pressure curves were obtained by recording the amplified output of Statham PM5TC transducer on storage oscilloscope. Occlusion time was limited to 0.25 since the occluder was automatically released by circuit which was activated by the negative pressure itself. This system is completely noiseless. Air flow monitored with Fleisch pneumotachometer placed between the mouth piece and the two-way valve assured that no flow occurred during occlusion. Mouth occlusion pressures were measured randomly 4 times during each measurement period at no less than 30 intervals. The inspiratory time was measured from the zero pressure points of three breaths just prior to each occluded breath.

Pressure, flow CO_2, and volume (from gasometer) were recorded on an Elema-Schönander Mingograph recorder.

output response during resistive loading would be increased more by a hypoxic stimulus than by a hypercapnic stimulus which elicits the same increase in ventilation in the absence of added resistance ("equivalent stimulus") To test this hypothesis the present study was designed to measure and compare the responses to hypoxia and to hypercapnia both in terms of minute ventilation (V_E) and in terms of mouth occlusion pressure, *i.e.* mouth pressure after the first 0 1 s of an occluded inspiration ($P_{0.1}$) This measurement reflects the force of approximately isometric contraction of inspiratory muscles and therefore the neural output to these muscles (Whitelaw *et al* 1975)

Methods

3 healthy young adults well trained as subjects in respiratory experiments were used in this study The two male subjects were 30 (TH) and 26 (HM) years old, the one female (BS) was 25 years old. For purposes of obtaining informed consent the experimental conditions were described to all subjects. They were not made aware of the aims of the experiments and were not told which type of experiment was to be done on a given day These steady-state experiments were begun at 9 AM with the subject comfortably seated in a semireclining position. They read material of their own choice during the experiments. Hypoxic and hypercapnic gas mixtures were delivered from pre-mixed tanks via two flowmeters for adjustments of the final mixture and were humidified before collection in a bag reservoir for delivery to a low resistance two-way valve Expired air was passed through a dry gasmeter or into Douglas bags for volume measurements and gas analysis.

All experiments were done in the same way and were of 4 types hypoxia without added resistance (R), *hypoxia with R CO_2 without R CO_2 with R. Only one type of experiment was done each day and consisted* of 3 steady-state periods of 15–20 min separated by 5–10 min rest periods. The following sequence was used in all experiments. atmospheric air first level of test gas, second level of test gas. Ventilation and endtidal CO_2 were continuously monitored. Data points were established from measurements made during intervals of 2 5 min at the end of each steady state period. A total of 49 expts. of all types were performed In all studies with hypoxia, CO_2 was included in the mixture to maintain a normal end tidal CO_2 Mean end-tidal CO_2 tensions (± 1 S.D) in the 3 subjects were 39.39 (± 0.48), 39 41 (± 0.96) mmHg and 39.13 (± 0.60) with R and 39.69 (± 0.50), 39 45 (± 0.69) and 39.55 (± 0.57) without R. Experiments with hypercapnia were performed in the same way with inspired CO_2 regulated to obtain steady-state hyperventilation at two levels approximating the two levels obtained with hypoxia Alveolar O_2 tension was kept between 150 and 200 mmHg during the experiments with hypercapnia.

Resistive loading was with porous discs warmed to prevent condensation and interposed between the subject and the two-way valve thus imposing added resistance during both phases of the respiratory cycle The resistance of the discs was 25 cm H_2O/l/s and linear between 0.2 and 1.0 l/s. In hypoxic experiments with R were the alveolar P_{O_2} and end tidal CO_2 (see above) adjusted to the same level as in experiments without R. From experience with our previous experiments it became possible to mix gases to obtain the desired ventilation and alveolar gas concentrations. Thereafter the experiments were done in random sequence over a period of approximately 8 months.

Inspired and expired gas samples were analyzed either by the micro Scholander method or with Beckman Model LB-1 CO_2 analyser and Servomex oxygen analyser The latter instruments were calibrated with gases of known concentration. End-tidal CO_2 tension ($P_{ET_{CO_2}}$) was calculated from the end of expiratory plateau of the record from a Beckman infra-red CO_2 analyser Sampling was carried out by microsampler from the external airway distal to the resistor discs and corrections were made in V_E for gas removed through the pump. The CO_2 analyser was calibrated with gases of known CO_2 concentrations immediately before and immediately after each observation period. Using $P_{ET_{CO_2}}$ in the Bohr formula, as a substitute for $P_{a_{CO_2}}$, dead space (V_D) was calculated. Assuming the same V_D for O_2 and CO_2 alveolar oxygen tensions ($P_{A_{O_2}}$) were calculated from the Bohr formula. All calibration gas mixtures were analyzed by the micro Scholander method With simultaneous analysis of arterial and arterialized capillary blood along with end-tidal and alveolar gases, we have shown that the added resistance does not change alveolar-arterial P_{O_2} gradient or end-tidal-arterial P_{CO_2} gradient (Barnett and Rasmussen 1970).

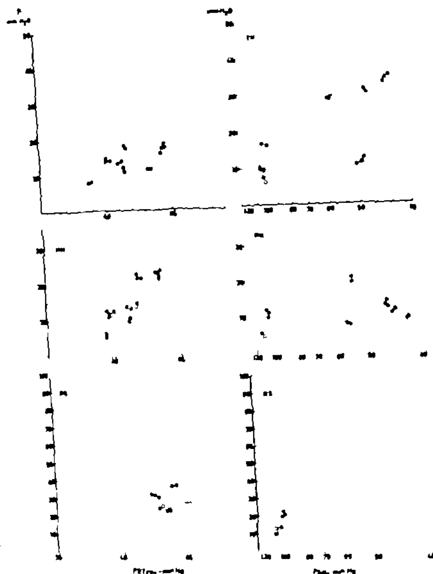


Fig. 2. Occlusion pressure responses to CO_2 (left) and to hypoxia (right). Open circles represent measurements without added resistance, closed circles measurement with added resistance.

normal atmospheric air the mean P without loading in subjects TH, HM and BS was 8.4, 6.3, and 11.9 mmH_2O respectively. With the increase in airway resistance that was used in these studies, these mean values on atmospheric air were approximately doubled and the difference was highly significant. With hypercapnia the P values also tended to be higher with than without resistance for any level of $P_{ET_{CO_2}}$. With hypoxia P was definitely higher with than without R at all levels of hypoxia. Linear regressions were calculated as

TABLE I Analysis of slopes of ventilatory responses to hypercapnia and hypoxia.

Subject	No added resistance		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef.	
$\Delta\dot{V}_E/\Delta P_{ET_{CO_2}}$					
TH	1 409	0 935	0.537	0 740	< 0 001
HM	1.503	0 870	0 477	0.809	< 0 001
BS	1 110	0.879	0.585	0.924	< 0.001
$\Delta\dot{V}_E/\Delta 1/(P_{A_{O_2}}-32)$					
TH	71.85	0.801	65.23	0.839	NS
HM	85.36	0 873	60.89	0.909	< 0.05
BS	95 48	0.725	97 42	0.898	NS

Expiratory reserve volumes (ERV) as well as vital capacities (VC) were measured in an add tional 5 expts. duri g hypoxia and 6 expts. during hypercapnia. For this purpose the subjects ere in a olume displacement body plethysmograph with box gas vol me changes being monitored with a Krogh spirometer The bo was tilted so that the subject was in a semireclining position similar to that used in the other experiments. After interposi g the resistor the subjects were then allowed to breathe either hypoxic or hypercapnic mi tures for 15 min and measurements of $P_{ET_{CO_2}}$ V_E and mouth occlusion pressures ere again made before nd after the collection periods as described above The final measurements were made 5 min fter remo l of the resistors but w th the inspired gas remaining the same I one e periment in each subject ERV was measured in a simila fashio while breathing air duri g hypo ia without resistance and 10 min fter the period of hypoxia.

Statistical tests were done using standard programs for Compucorp 327 computer which ga e mean, SD SE, regressionline coeffic e ts a and b, and correlation coefficient r Tests of significance of differences between regression lines were done by the method described by Hald (1952).

Results

The ventilatory responses to hyperoxic hypercapnia and to eucapnic hypoxia in the three subjects are shown in Fig 1 In order to facilitate visual comparison the 2 sets of data for each subject are placed together and the $V_E - P_{A_{O_2}}$ relationship has been plotted on semilogarithmic paper with $P_{A_{O_2}}$ values decreasing from left to right. It is apparent that the ventilatory response to CO was reduced in all subjects with resistive loading when compared with that in the unloaded state. When ventilation was plotted against $P_{A_{O_2}}$ a reduction in the response in the presence of increased resistance was not so apparent In Table I are shown the results of analyses of these data The slopes and correlation coefficients for linear regressions of the relationship between $P_{ET_{CO_2}}$ and V_E are shown. The reduction in slopes with increased airway resistance is highly significant in all subjects. In order to analyse the relationship between P_{A_O} and V_E as a linear function the term $1/(P_{A_{O_2}}-32)$ was used as suggested by Byrne-Quinn *et al* (1972) Analysis of V_E versus this term shows no difference in the slopes in two subjects and the decrease in slope with added resistance in subject HM is barely significant.

Fig. 2 presents in a similar way the responses to hypercapnia and to hypoxia expressed as mouth occlusion pressure 0 1 s after beginning an occluded inspiration (P) As with the ventilatory response there was rather wide scatter of points. For all measurements on

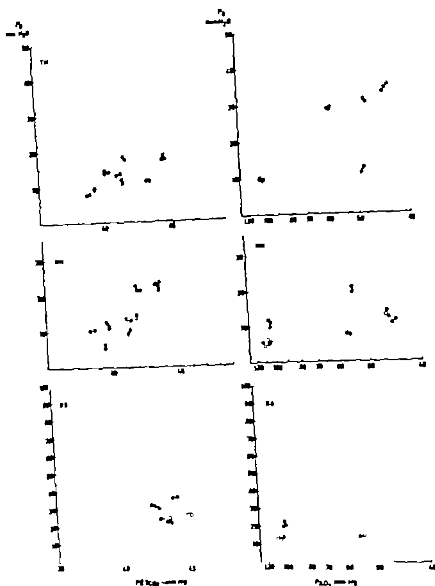


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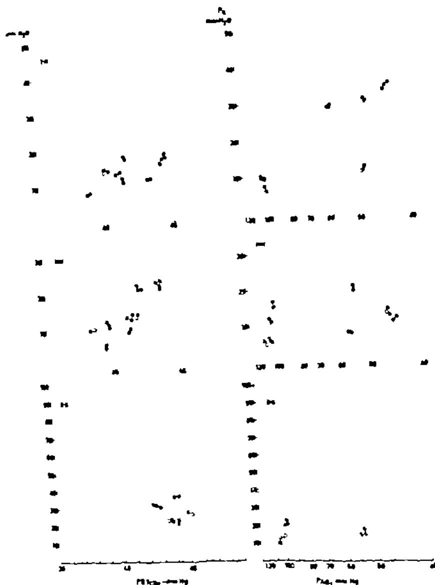


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Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P_{.1}/\Delta P_{ET_{CO_2}}$					
TH	1.307	0.830	0.799	0.480	NS
HM	1.193	0.697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
$\Delta P_{.1}/\Delta 1/(P_{A_{O_2}} - 32)$					
TH	81.00	0.697	13.40	0.668	<0.001
HM	77.69	0.681	167.00	0.852	<0.01
BS	176.90	0.701	912.70	0.904	<0.001

described above Testing of the difference in slope (Table II) shows what is apparent from the figures, *i.e.* the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load. In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia.

Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO_2 breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of $P_{ET_{CO_2}}$ studied were very similar to those over the range of hypoxia. This applied to all three subjects, although the P values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted

In the absence of a resistive load the increase in V_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (f) There was no significant difference in any subject between the slope *of the relationship V_E/V_T during hypercapnia from that during hypoxia. With resistance* added f was always reduced but the relationship V_E/V_T was the same during hypoxia and hypercapnia in two of the subjects (Fig. 3) In subjects BS there was an increase in both f and V_T when hyperventilation resulted from hypoxia.

Inspiratory time (T_I) was always increased with added resistance The increase varied from 45% to 61% above the T_I without a resistive load in subjects HM and TH and the difference was very similar with both hypercapnia and hypoxia In subject BS the mean increase in T_I with loading in association with hypercapnia was 32% but with hypoxia it was only 10%. The ratio of inspiratory time to total respiratory cycle time (T_t) was remarkably similar under all experimental conditions. In subjects TH and HM the T_I/T_t ratios were slightly higher with than without resistance, but this difference was not significant

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40.8–53.9 Avg. −46.5 mmHg) and in 6 expts. with hypercapnia

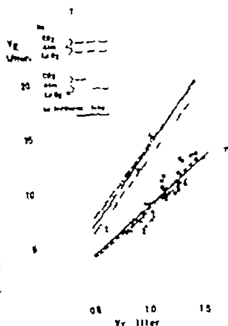


Fig. 3. Relationship between minute ventilation and tidal volume during hypoxia and during hypercapnia—with and without added airway resistance in subject TH.

P_{ETCO_2} range 43.9–46.8, Avg. ~45.1 mmHg). The ERV was always greater with than without added resistance but there was no significant change in vital capacity. The magnitude of the change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances the ERV returned to normal within 5 minutes after removal of the resistor even though the inspired hypoxic or hypercapnic gas mixture remained the same. In one experiment in each subject VC and ERV were measured before, during and after a 20 minute period of hypoxia ($P_{A_{O_2}}$ 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

It was the aim of this study to compare the effects of resistive loading on the responses to hypoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses in terms of mouth occlusion pressure which is a more meaningful indicator of neural output. The results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the unloaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$).

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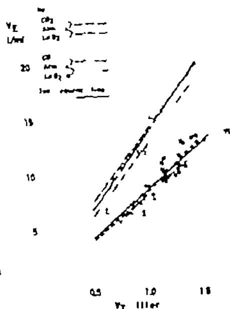
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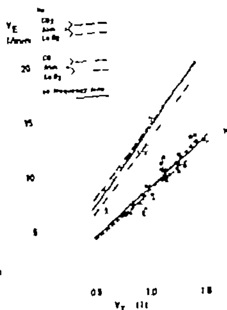


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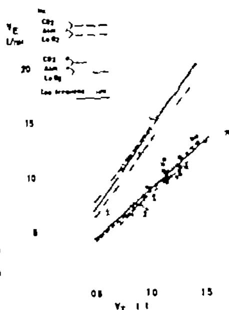
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When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted

In the absence of a resistive load the increase in V_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (f) There was no significant difference in any subject between the slope of the relationship V_E/V_T during hypercapnia from that during hypoxia. With resistance added f was always reduced but the relationship V_E/V_T was the same during hypoxia and hypercapnia in two of the subjects (Fig. 3) In subjects BS there was an increase in both f and V_T when hyperventilation resulted from hypoxia

Inspiratory time (T_I) was always increased with added resistance. The increase varied from 45% to 61% above the T_I without a resistive load in subjects HM and TH and the difference was very similar with both hypercapnia and hypoxia In subject BS the mean increase in T_I with loading in association with hypercapnia was 32%, but with hypoxia was only 10% The ratio of inspiratory time to total respiratory cycle time (T_I) was remarkably similar under all experimental conditions. In subjects TH and HM the T_I/T ratios were slightly higher with than without resistance, but this difference was not significant.

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40 8–53 9 Avg. −46.5 mmHg) and in 6 expts. with hypercapnia



Relationship between minute ventilation and ... during hypoxia and during hyper... with and without added airway resistance in TH

...range 43.9–46.8, Avg. –45.1 mmHg). The ERV was always greater with than with... ...dded resistance but there was no significant change in vital capacity. The magnitude ... change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ... HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances ...RV returned to normal within 5 minutes after removal of the resistor even though the ...red hypoxic or hypercapnic gas mixture remained the same. In one experiment in ... subject VC and ERV were measured before, during and after a 20 minute period of ...xia ($P_{A_{CO_2}}$ 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

...as the aim of this study to compare the effects of resistive loading on the responses to ...xia and hypercapnia. Since minute ventilation does not reflect neural output to inspira... muscles in the presence of mechanical abnormalities, we have also measured responses ...rms of mouth occlusion pressure which is a more meaningful indicator of neural output. ... results show that in the presence of resistive loading ventilation was better maintained ...ng hypoxia than during levels of hypercapnia which yielded similar responses in the ...oaded state. This was accomplished by a relatively greater force output of the respiratory ...cles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$).

...ecause of differences in the time required to reach maximum response for respectively ...oxia and hypercapnia, a steady-state method has been used for studying the responses ... was done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier ...es of experiments resistive loading led to some reduction in the ventilatory response to

TABLE II Analysis of slopes of occlusion pressure responses to hypercapnia and hypoxia.

Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P_{.1}/\Delta P_{ET_{CO_2}}$					
TH	1 307	0.830	0.799	0.480	NS
HM	1 193	0 697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
$\Delta P_{.1}/\Delta 1/(P_{A_{O_2}}-32)$					
TH	81.00	0.697	213 40	0.668	<0.001
HM	77 69	0.681	167 00	0.852	<0.01
BS	176.90	0.701	912.70	0.904	<0.001

described above. Testing of the difference in slope (Table II) shows what is apparent from the figures, *i.e* the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia

Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of $P_{ET_{CO_2}}$ studied were very similar to those over the range of hypoxia This applied to all three subjects, although the P values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted

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Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40 8–53.9 Avg. – 46.5 mmHg) and in 6 expts. with hypercapnia

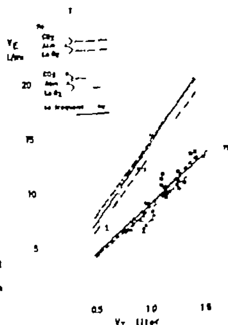


Fig. 3. Relationship between minute ventilation and tidal volume during hypoxia and during hypercapnia—with and without added airway resistance in subject TH

(P_{ETCO_2} range 43.9–46.8, Avg. ~45.1 mmHg). The ERV was always greater with than without added resistance but there was no significant change in vital capacity. The magnitude of the change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances the ERV returned to normal within 5 minutes after removal of the resistor even though the inspired hypoxic or hypercapnic gas mixture remained the same. In one experiment in each subject VC and ERV were measured before, during and after a 20 minute period of hypoxia (P_{AO_2} 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

It was the aim of this study to compare the effects of resistive loading on the responses to hypoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses in terms of mouth occlusion pressure which is a more meaningful indicator of neural output. The results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the unloaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$).

Because of differences in the time required to reach maximum response for respectively hypoxia and hypercapnia, a steady-state method has been used for studying the responses as was done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier series of experiments resistive loading led to some reduction in the ventilatory response to

eucapnic hypoxia but in all 3 subjects an appreciably greater reduction was seen in the response to normoxic hypercapnia. The resistor used in the present studies had a more linear pressure flow relationship and the resistance was less at higher flow rates, than that used in the earlier experiments Only one (HM) of the 3 subjects in the recent experiments showed slight reduction in the ventilatory response to hypoxia with 25 cm H_2O/l/s added resistance. The response to hyperoxic hypercapnia, however was significantly and markedly reduced in all subjects Doell *et al* (1973) found no reduction in the ventilatory response to hypoxia in 6 subjects with a smaller increase in resistance. They did find a reduction in response to hypercapnic hypoxia at 4 ATA and Rebuck and Juniper (1975) described a reduced ventilatory response to hypercapnic hypoxia with 20 cm H_2O/l/s of inspiratory resistance No direct comparison with normoxic or hyperoxic hypercapnia was reported The results of these various experiments lead us to the conclusion that, whereas the hypoxic ventilatory response may obviously be reduced with sufficiently high resistive loads, ventilation is maintained at more nearly normal levels with moderate added resistance in association with hypoxia than when an equal resistive load is added during hypercapnia

It was assumed earlier (Barnett and Rasmussen 1970) that a hypercapnic stimulus—and a hypoxic stimulus which resulted in similar ventilation were physiologically equivalent stimuli although it was recognized that alterations in resistance within the subject s airways as a result of one or both of these conditions might invalidate this assumption In the present experiments without R the values for mouth occlusion pressure for any level of ventilation were identical regardless of whether the stimulus was hypoxia or hypercapnia This observation makes it unlikely that differences in subjects airways resistance were present In so far as the P is an indicator of the neural output to inspiratory muscles, it offers more convincing evidence that the two conditions were physiologically equivalent as respiratory stimuli

Assuming that mouth occlusion pressure reflects the force output of inspiratory muscles, then the increment in this force was greater during hypoxia than during hypercapnia when external resistance was added However the presumption that a greater occlusion pressure reflects a greater neural output to inspiratory muscles is not justified if end tidal lung volume changes (Marshall 1962) In seated subjects Fitzgerald *et al* (1976) found variable but similar increases in FRC with short exposure to hypoxia (12% O_2) and hypercapnia (4% CO_2) Kellogg and Mines (1975) failed to demonstrate a change in FRC in seated or recumbent subjects when alveolar oxygen tensions were quickly lowered to 42–50 mmHg. The expiratory reserve volumes (ERV) were consistently increased in all 3 of our subjects in association with the resistive load, a finding which agrees with that reported by Zechman *et al* (1957) This change was not marked and was the same with hypoxia and hypercapnia. Furthermore, it was reversed by removal of the resistor while inspired gas concentrations remained unchanged. Comparable levels of hypoxia without added resistance failed to alter ERV Earlier experiments on other normal subjects under almost identical conditions (Barnett, unpublished data) showed no effect of hypercapnia alone on these lung volumes. Thus, we were unable to explain the difference in effect of resistive loading on ventilatory and occlusion pressure responses with hypoxia and hypercapnia by differences in lung volumes. However it is possible that the thoraco-abdominal configuration and the diaphragmatic muscle length may have changed without alteration in the end-tidal volume.

The patterns of breathing during hypoxia and hypercapnia without added resistance were similar in all subjects, i.e. hyperventilation resulted mainly from an increase in tidal volume with little change in frequency. With resistive loading respiratory frequency was reduced and in 2 subjects (TG and HM) this effect was the same with hypoxia and hypercapnia, in one subject (BS) the increase in ventilation with hypoxia resulted from an increase in both frequency and tidal volume. We have reexamined data from 3 subjects previously reported (Barnett and Rasmussen 1970) and in all instances the relationship between V̇ and V̇ was linear and similar during hypoxia to that during hypercapnia. Thus in 5 of 6 subjects the breathing patterns were as depicted in Fig. 3. The difference in the effect of resistive loads on the ventilatory and P responses to hypoxia and hypercapnia, therefore, cannot be explained by difference in breathing patterns.

An increase in mouth occlusion pressure in association with inspiratory resistive loading was demonstrated by Kryger *et al.* (1975) and subsequently by Altose *et al.* (1976) and Kelsen *et al.* (1976). This increase was attributed to an increase in neural output. They suggest that this is mediated through a "higher" possibly cortical, mechanism since the increase in P in goats with inspiratory loading during hypercapnia was almost entirely eliminated by sodium pentothal anesthesia (Isaza *et al.* 1976). Subsequently similar results have been reported in anesthetized human subjects (Whitelaw *et al.* 1976). At present the exact nature of this increase in ventilatory drive in the presence of resistive loading is unknown. It would appear however that it is of greater magnitude with hypoxia than with hypercapnic stimulus which produces an equivalent ventilatory response in the absence of the resistive load. The data suggest a multiplicative interaction between hypoxia and resistive loading in determining the neural output to inspiratory muscles. This situation is reminiscent of that which occurs with exercise where in both animals (Kao *et al.* 1967) and man (Asmussen 1967) the exercise stimulus to ventilation has been found to interact with hypoxia in more than an additive manner. Breathing against resistance means that the respiratory muscles are in a state of exercise. It seems likely that these "exercising" muscles would influence ventilation as does muscular exercise otherwise and that this influence might be greater during hypoxia than during hypercapnia. Flenley *et al.* (1970) found normal or increased ventilatory responses to hypoxia in 7 of 9 patients with cronic obstructive disease (COPD) who showed reduced responses to CO. The results of the present study may contribute to the understanding of these findings and may help to explain the importance of the hypoxic stimulus in maintaining ventilation in the presence of obstructive airways disease.

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Nervous control of pancreatic exocrine secretion in pigs

By

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Abstract

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The pancreatic secretion of fluid, bicarbonate and protein in response to electrical stimulation of the vagus and splanchnic nerves, to exogenous and endogenous secretin and to various pharmacological agents was studied in anesthetized young pigs (21 kg). Vagal stimulation increased flow, bicarbonate output and protein output in a frequency dependent manner; the half maximal effective frequency was 2–4 Hz and the maximal effective frequency 12 Hz. The secretory response to vagal stimulation was potentiated by physiological elevations of the arterial concentration of secretin brought about by injection of secretin or by acidification of the duodenal bulb. Simultaneous stimulation of the splanchnic nerves strongly inhibited the response to vagal stimulation; splanchnic nerve stimulation alone had no demonstrable effect. The flow and bicarbonate response to vagal stimulation was unaffected by atropine, but abolished by hexamethonium. Protein output was strongly inhibited by both agents. The response to intraarterial infusion of acetylcholine resembled that elicited by vagal stimulation but it was smaller and it was completely abolished by atropine and unaffected by hexamethonium. Alpha- and beta-adrenergic blockade stimulated rather than inhibited the secretory response to vagal stimulation. The portal vein plasma concentration of secretin was not affected by vagal stimulation. The results indicate that the protein response, and the flow and bicarbonate response to vagal stimulation are not brought about by the same mechanism. An increased release of secretin is not involved. Peptidergic (VIP-containing) nerves may contribute.

The control of pancreatic secretion of fluid and bicarbonate is generally believed to be exerted chiefly by changes in the concentration of circulating secretin (Harper 1972). Radioimmunological determination of secretin concentrations in plasma have confirmed, that instillation of acid into the small intestine is associated with increasing secretin concentrations, and that the resulting secretin concentrations when reproduced by exogenous secretin administration may explain a major part of the pancreatic secretory response to acidification (Schaffalitzky de Muckadell *et al.* 1977 a, Rhodes *et al.* 1976, Isenberg *et al.* 1977, Häcki *et al.* 1977, Schaffalitzky de Muckadell *et al.* 1978).

It has also recently been established that the gastric emptying of acid into the duodenum and hence the duodenal pH are closely related to the concentrations of immunoreactive secretin in peripheral blood (Schaffalitzky de Muckadell and Fahrenkrug (in press)). Ingestion of a meal, however, is associated with only small and inconstant increases in peripheral

concentrations of secretin in man (Schaffalitzky de Muckadell and Fahrenkrug (in pres Rhodes *et al* 1976 Fahrenkrug and Schaffalitzky de Muckadell 1977) although the pancr. in this situation would be expected to contribute to the digestive processes by a great increased production of juice, bicarbonate and enzymes (the dimension of the fluid respon in man remains undocumented but in dogs there is a pronounced fluid response to feedi. (Llanos *et al* 1977) If such a secretion takes place, it could not be due to the small rise i the secretion of secretin alone (Schaffalitzky de Muckadell *et al* 1978 Llanos *et al* 1977 The pancreas of man and pig has been reported to receive extensive cholinergic innervatio (Comline *et al* 1964), and the pig has been reported to respond to vagal stimulation wi profuse exocrine secretion (Hickson 1970 b). In other species, the volume and bicarbonat response to vagal stimulation is usually small (Thomas 1967 Preshaw 1967 Sjödin 1977 and other investigators were not able to confirm the above mentioned impressive fluid an bicarbonate response in pigs (Magee and White 1965)

We therefore decided to investigate, in anaesthetized pigs, the role of the autonomi nervous system in the control of pancreatic exocrine secretion and have attempted to identify the mechanisms by which this control is exerted.

Using the recently developed radioimmunoassay for secretin, we also evaluated the possible role of this hormone in the pancreatic response to vagal stimulation

Materials and methods

I *Animal and operative procedure*

50 young female Danish Landrace pigs (weight 17 25 kg, media 1 kg) were obtained from the same breeder Food but not water which cont ined 10 glucose was withdrawn 18 h before experiments

Azaperone (Sedaperone J nssen Pharmaceutica Beerse Belgium) 4 mg kg⁻ intramuscularly was used f r premedication A esthesia was induced with halothane/N O/O and maintained with chloralose 70 mg kg⁻ intra enously After nsertion of an endotracheal cuffed tube termittent positive pressure ventilation was maintained by a Bennet nesthesia ventilator in a semi-open system. Polyethylene catheters were inserted into the left femoral artery and ein. Intraarterial blood pressure and rectal temperature wa continuously monitored The animal were kept w rm by means of an lectric heating pad The sto mach the duodenum, nd the pancreas were exposed through mid-line incision. A polyethylene catheter (dead space 0.2 ml) was inserted int the pancreatic duct close t its entrance to the duodenum. The gastro-pyloric junction was closed by me ns of a l gature around the pylorus, carefully placed a close to the muscula wall as possible to avoid damage to vessels and erves. The gastric co tents were cont nuously drained by an oro-gastric tube (Ch 30).

II *Experimental protocol*

a) *Basal secretion* Pancreatic juice w collected for 15 min periods begin ing 60 minutes after the com pletio of the operative procedure

b) *Pancreatic xocrine response t secretin* 39 pigs received pure natural porcine secretin (GIH Research Laboratory Karolinska Institutet, Stockholm, Sweden) 0.5 Clinical Units kg i v as a bolus. This dose produced for more than 5 min plasma concentrations of secretin above that eliciting maximal secretion of fluid and bicarbonate (*Schaffalitzky de Muckadell et al* 1977). *Pancreatic juice* w s collected for three 5-min periods, nd a subseque t 15-min period. In 8 e pt J ice was collected for 5 min, 25 t 30 min after the secretin injection I 10 pigs blood samples for secretin determination were draw from the portal vein 30 min after the admi istration of secretin.

c) *Vagal stimulation*. After thoracotomy the vagal trunci were identified above the diaphragm, and cut below the heart. The peripheral cut ends were threaded through a bipola platinium electrode (Dale and Henderson 1967)

Stimulation was performed with constant current square-wa e impulses (impulse duration 4 ms, 8 mA)

or 5 min (delivered by an Electronic Square-wave Stimulator Palmer U.K.). Applied frequency was varied between 0.25 and 40 Hz. Pancreatic juice was collected for three 5-min periods during and after the stimulation, when secretin had previously been administered at least 60 min elapsed before stimulation. In 25 expts. In 17 pigs blood samples for secretin determination were obtained from the portal vein −15, −10, −5, 0, ½, 1, 3, 5, 10, and 15 min after the start of stimulation. In 2 expts. blood samples were also obtained from the femoral artery during vagal stimulation.

d) Potentiation by secretin of the response to vagal stimulation. (1) Exogenous secretin. Vagal stimulations were carried out 30 min after the administration of secretin as described in section b, and compared to similar stimulations more than 60 min after secretin.

(2) Endogenous secretin. In 4 pigs vagal stimulation was performed during simultaneous perfusion of the duodenal bulb with acid. Two soft catheters (Ch 8) were mounted in a rubber stopper which was introduced into the duodenum through a small distal duodenostomy and anchored 3 cm from the pylorus by means of a ligature. The duodenal bulb was then perfused with 1) citrate buffer, 60.4 mmol/l and adjusted to pH 4 with HCl, and 2) citrate buffer 5 mmol/l, adjusted to pH 1 with HCl. Both buffers were made isotonic with NaCl, and heated to 37°C before perfusion. By titration to pH 7 both buffers were found to have a titratable acidity of 100 mmol/l. Perfusion was carried out at a flow of 30 ml/min, which was maintained for 25 min. After 20 min the vagus nerves were stimulated at 4 Hz. Pancreatic juice was collected for two 10 min periods and one 5 min period. Blood samples for secretin determination were obtained as described in section.

e) The splanchnic nerves. (1) The splanchnic nerves were cut just below the diaphragm in all expts. (section e–g) except 5 in which the splanchnic nerves were dissected free; a ligature was placed loosely around the nerves, and threaded through a stiff tube which was exteriorized through the flanks of the animal. The vagal stimulation was carried out as above, and repeated after the splanchnic nerves had been severed by traction of the ligature. Pancreatic juice was collected as described in section c.

(2) Splanchnic nerve stimulation. The left splanchnic nerves were dissected free just below the diaphragm, cut, and the distal end threaded through a bipolar platinum electrode. Stimulation was carried out using a second stimulator (impulse duration 1 ms, 5 mA, 10 Hz for 5 min). The right splanchnic nerves were cut. Pancreatic juice was collected as described in section c.

f) Reproducibility and fatiguability of response. In 7 pigs 5 min vagal stimulations were repeated 2–4 times over 3 h. In 6 pigs vagal stimulation was continued for 30 min.

g) Pharmacological experiments. The pancreatic response to vagal stimulation was studied after blockade with atropine in various doses (0.005 to 2 mg/kg b.wt., as atropine sulphate). Atropine was also administered as a primed infusion (0.5 mg/kg initially followed by 1 mg h^{-1} kg^{-1}) in 2 expts. Hexamethonium (Sigma Chemical Company St. Louis, Mo., U.S.A.) was given at 10–30 mg kg^{-1} (as hexamethonium bromide). Propranolol (Inderal® I.C.I. U.K.) was given as a dose of 1 mg kg^{-1}. Phenoxybenzamine (Alfred Benzon, Copenhagen) was given as a slow infusion, in a total dose of 1 mg kg^{-1}. For studies with acetylcholine (Sigma Chemical Company), a catheter was introduced via the right femoral artery into the aorta with its tip 5 cm above the outlet of the celiac artery. The acetylcholine was dissolved immediately before use in saline and infused by means of a constant infusion syringe pump (Unita, Braun-Melsungen, GFR). Acetylcholine was infused for 5 min, and pancreatic juice was collected as during vagal stimulation (section c). In two pigs a step-dose experiment was carried out, with doses ranging from 0.05 to 100 mg/min for 5 min. Pancreatic juice was collected for 5 min periods.

Laboratory analyses

Volume of the pancreatic juice was recorded to the nearest 0.01 ml. Bicarbonate and protein concentration were determined as previously described (Schaffalitzky de Muckadell *et al.* 1977).

Blood samples for secretin determination were drawn into chilled tubes containing heparin and aprotinin (Trasylol Bayer GFR) 500 KIU/ml blood. Plasma secretin concentrations were determined radioimmunochemically (Schaffalitzky de Muckadell *et al.* 1977 a, Schaffalitzky de Muckadell and Fahrenkrug 1977 c).

Calculations

The results concerning exocrine secretion are presented as follows. Flow of juice ml h^{-1} concentration of protein in pancreatic juice g/l, output of protein g h^{-1} concentration of bicarbonate mmol/l and output of bicarbonate mmol h^{-1}. The data are presented as the median followed by the 95 % confidence limits of the median, or the total range for n < 8 (Documenta Geigy 1968). Where two responses from the

same animal are compared the results are expressed in % of the previous response, and medians as above. Statistical evaluation was performed using the Mann-Whitney U-test, The Wilcoxon matched-pairs signed-ranks test, and The Friedman two-way analysis of variance (Siegel 1956).

Results

a) *Basal secretion*

There was a small basal secretion of juice in all animals, but the volume secreted was often too low compared to the dead space of the pancreatic catheter to allow bicarbonate and protein determination Basal flow was recorded for 21 animals, and protein and bicarbonate concentrations determined in 8 samples. The results appear in Table I

b) *Pancreatic exocrine response to exogenous secretin*

(Table I) An increased flow of juice was noted 30 to 40 s after the administration of secretin in all pigs. Stimulated secretion was maintained for at least 10 min. Compared to basal levels, flow was increased 102 times and bicarbonate concentration rose from 69 mmol/l to 143 mmol/l The concentration of protein fell rapidly from the basal level of 8.7 g/l to 0 4 g/l whereas protein output increased during the first 5 min period and then gradually fell below basal output Twentyfive to 30 min after administration of secretin the flow of juice was still slightly but significantly elevated.

c) *Vagal stimulation*

The results of maximal or supramaximal stimulations (12–20 Hz) in 29 pigs are listed in Table II The flow of juice increased 40–50 s after the start of stimulation in all pigs. Increased secretion could be maintained for as long as the electrical stimulation was continued (Table IX) but the secretion declined rapidly after cessation of stimulation and approached basal levels (or less) after 10 min During the first 5 min of stimulation the flow of juice

TABLE I Pancreatic exocrine response to secretin.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Basal secretion	0.56 (0.40–0.92) n = 21	8 7 (4 0–29.6) n = 8	69 (45–110) = 8	0.011 (0.009–0.053) n = 8	0.080 (0.032–0 23) n = 8
Response to bolus injection of 0.5 CU/kg at 0 min (= 39)					
0–5 min	57 (48–66)	*5.9 (4.1 10.9)*	*131 (123–134)*	*0.29 (0.23–0.46)*	*7 6 (6.1 9 0)*
5–10 min	53 (48–60)	0.4 (0.4–0.6)	138 (136–144)	0.024 (0.019–0 034)	7 1 (6.7–8.8)
10–15 min	36 (30–42)	0.4 (0.2–0.5)	143 (142 146)	0.009 (0.006–0.016)	5 4 (4.3–6.5)
15–30 min	9 (8–12)	0.5 (0.2–0.7)	130 (126–134)	0 004 (0 002–0 006)	1 07 (0.99–1 61)
25–30 min	2.1 (0.5–3.6)[a]	—	—	—	—

The data are presented as the median followed by the 95 % confidence limits of the median.
This time interval was studied in 8 pigs only

TABLE II. Pancreatic exocrine response to 5 min supramaximal vagal stimulation.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Basal secretion	0.56 (0.40–0.92) n = 21	8.7 (4.0–29.6) n = 8	69 (45–110) n = 8	0.011 (0.009–0.053) n = 8	0.080 (0.032–0.23) n = 8
Time after start of stimulation min (n = 29)					
0–5	31 (24–42)	108 (90–140)	126 (120–132)	3.28 (2.30–3.85)	3.94 (2.45–5.46)
5–10	5.4 (3.9–6.6)	37 (25–61)	136 (128–140)	0.20 (0.13–0.24)	0.76 (0.59–0.89)
10–15	0.020 (0–8.120)	—	—	—	—

The data are presented as the median followed by the 95 % confidence limits of the median.

increased 52 times, the concentration of protein 12 times, the bicarbonate concentration 1.8 times and the output of protein and bicarbonate 300 and 50 times, respectively.

In 23 animals the exocrine response to both exogenous secretin and to vagal stimulation was studied. Thus the response to vagal stimulations (which were performed more than 60 min after the injection of secretin at which time the plasma concentration of secretin had returned to the preinjection level (not shown)) could be compared to the response to secretin alone with each animal as its own control. The flow of juice during supramaximal vagal stimulation amounted to 59 % (median, 95 % confidence limits 47–75 %) of that obtained in the second 5 min period after secretin. The bicarbonate output constituted 55 % (42–75 %) while the protein output during vagal stimulation was 9 100 % (6 900–20 200 %) of that obtained in the second 5 min period after secretin. In 4 of 23 animals the flow as well as the bicarbonate output exceeded that obtained after secretin, and in 7 and 9 respectively the flow and bicarbonate output constituted more than 70 % of that obtained after secretin.

TABLE III. Pancreatic exocrine response to vagal stimulation at various frequencies.

Frequency Hz[a]	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
0.25 (1)	1.2	—	—	—	—
0.375 (1)	4.8	2.7	45	0.13	0.22
0.5 (2)	2.1, 0.64	—	—	—	—
2 (8)	14.1 (5.4–46.0)	118 (38–217)	85 (62–134)	1.37 (0.38–5.70)	1.31 (0.43–6.2)
4 (11)	18.6 (12.0–26.4)	120 (70–189)	110 (96–124)	2.30 (1.39–3.73)	1.90 (1.32–3.22)
8 (16)	31.0 (25–49)	116 (69–162)	124 (112–130)	3.96 (2.41–6.15)	3.90 (2.72–6.10)
12 (8)	43.3 (18.0–74)	101 (54–138)	133 (110–140)	3.63 (2.01–5.96)	5.62 (2.16–9.97)
20 (22)	27.0 (18.2–37.0)	108 (90–140)	124 (118–132)	3.14 (2.18–3.61)	3.26 (2.16–4.97)
40 (1)	24.0	46	124	1.10	2.97

[a] Vagal stimulation was carried out for 5 min. The values are derived from analysis of the secretory response during these 5 min. Number of pigs in brackets in the first column. The data are presented as the median followed by the 95 % confidence limits of the median. The effect of 4 Hz stimulation was studied in 11 expts. in 5 pigs.

same animal are compared, the results are expressed in % of the previous response and as medians as above Statistical evaluation was performed using the Mann Whitney U test, The Wilcoxon matched-pairs signed-ranks test, and The Friedman two-way analysis of variance (Siegel 1956).

Results

a) *Basal secretion*

There was a small basal secretion of juice in all animals, but the volume secreted was often too low compared to the dead space of the pancreatic catheter to allow bicarbonate and protein determination. Basal flow was recorded for 21 animals, and protein and bicarbonate concentrations determined in 8 samples. The results appear in Table I.

b) *Pancreatic exocrine response to exogenous secretin*

(Table I) An increased flow of juice was noted 30 to 40 s after the administration of secretin in all pigs. Stimulated secretion was maintained for at least 10 min. Compared to basal levels, flow was increased 102 times and bicarbonate concentration rose from 69 mmol/l to 143 mmol/L The concentration of protein fell rapidly from the basal level of 8.7 g/l to 0 4 g/l whereas protein output increased during the first 5 min period and then gradually fell below basal output. Twentyfive to 30 min after administration of secretin the flow of juice was still slightly but significantly elevated.

c) *Vagal stimulation*

The results of maximal or supramaximal stimulations (12–20 Hz) in 29 pigs are listed in Table II The flow of juice increased 40–50 s after the start of stimulation in all pigs. Increased secretion could be maintained for as long as the electrical stimulation was continued (Table IX) but the secretion declined rapidly after cessation of stimulation and approached basal levels (or less) after 10 min During the first 5 min of stimulation the flow of juice

TABLE I Pancreatic exocrine response t secretin.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonat conc. mmol/l	Prot in output g/h	Bicarbonate output mmol/h
Basal secretion	0.56 (0.40–0.92) n = 21	8 7 (4 0–29.6) n = 8	69 (45–110) n = 8	0.011 (0 009–0.053) = 8	0.080 (0.032–0 23) = 8
Response to bolus injection of 0.5 CU/kg at 0 min (n = 39)					
0–5 min	57 (48–66)	5 9 (4 1 10.9)	131 (123–134)	0.29 (0.23–0.46)	7.6 (6.1–9 0)
5–10 min	53 (48–60)	0.4 (0 4–0.6)	138 (136–144)	0.024 (0.019–0.034)	7 1 (6.7–8 8)
10–15 min	36 (30–42)	0.4 (0.2–0.5)	143 (142 146)	0.009 (0 006–0.016)	5 4 (4.3–6.5)
15–30 min	9 (8–12)	0.5 (0.2–0.7)	130 (126–134)	0.004 (0.002–0.006)	1.07 (0.99–1 61)
25–30 min	2.1 (0.5–3.6)[a]	—	—	—	—

The data are presented as the median followed by the 95 % confidence limits of the median.
[a] This time interval was studied in 8 pigs only

TABLE II. Pancreatic exocrine response to 5 min supramaximal vagal stimulation.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Basal secretion	0.56 (0.40–0.97) 21	8.7 (4.0–29.6) = 8	69 (45–110) 8	0.011 (0.009–0.053) = 8	0.080 (0.032–0.23) = 8
Time after start of stimulation (... 23)					
0–5	31 (24–42)	108 (90–140)	126 (120–132)	3.28 (2.30–3.85)	3.96 (2.45–5.46)
5–10	5.4 (3.9–6.6)	37 (25–61)	136 (128–140)	0.20 (0.13–0.24)	0.76 (0.59–0.89)
10–15	0.020 (0–0.120)	—	—	—	—

The data are presented as the median followed by the 95 % confidence limits of the median.

increased 52 times, the concentration of protein 12 times, the bicarbonate concentration 1.8 times and the output of protein and bicarbonate 300 and 50 times, respectively

In 23 animals the exocrine response to both exogenous secretin and to vagal stimulation was studied. Thus the response to vagal stimulations (which were performed more than 50 min after the injection of secretin at which time the plasma concentration of secretin had returned to the preinjection level (not shown)) could be compared to the response to secretin alone with each animal as its own control. The flow of juice during supramaximal vagal stimulation amounted to 59% (median, 95% confidence limits 47–75%) of that obtained in the second 5 min period after secretin. The bicarbonate output constituted 55% (42–73%) while the protein output during vagal stimulation was 9 100% (6 900–20 200%) of that obtained in the second 5 min period after secretin. In 4 of 23 animals the flow as well as the bicarbonate output exceeded that obtained after secretin, and in 7 and 9 respectively the flow and bicarbonate output constituted more than 70% of that obtained after secretin.

TABLE III. Pancreatic exocrine response to vagal stimulation at various frequencies.

Frequency Hz[a]	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
0.25 (1)	1.2	—	—	—	—
0.375 (1)	4.8	2.7	45	0.13	0.22
0.5 (2)	2.1 0.66	—	—	—	—
2 (8)	14.1 (5.4–46.9)	118 (38–217)	85 (62–134)	1.37 (0.38–5.70)	1.31 (0.43–6.2)
4 (11)	18.6 (12.0–26.4)	120 (70–189)	110 (96–124)	2.30 (1.19–3.73)	1.90 (1.32–3.22)
8 (14)	31.0 (25–49)	116 (69–162)	124 (112–130)	3.96 (2.41–6.15)	3.90 (2.72–6.10)
12 (8)	43.3 (18.0–74)	101 (54–138)	133 (110–140)	3.63 (2.01–5.96)	5.62 (2.16–9.97)
20 (22)	27.0 (19.2–37.0)	108 (90–140)	124 (118–132)	3.14 (2.18–3.61)	3.26 (2.16–4.97)
40 (1)	24.0	46	124	1.10	2.97

[a] Vagal stimulation was carried out for 5 min. The values are derived from analysis of the secretory response during these 5 min. Number of pigs in brackets in the first column. The data are presented as the medians followed by the 95 % confidence limits of the median. The effect of 4 Hz stimulation was studied in 11 expts. in 5 pigs.

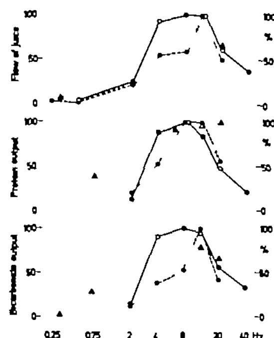


Fig. 1 Individual values for flow of juice, protein output, and bicarbonate output expressed as per cent of the maximum values obtained during vagal stimulation at various frequencies in three pigs.

In another series of expts. the responses to various frequencies of stimulation were studied (Table III). In 3 out of 4 expts. with frequencies below 1 Hz, the flow increased during stimulation. The half-maximal effective frequency was between 2 and 4 Hz and maximal stimulation of flow occurred at 12 Hz. Higher frequencies appeared less effective. The maximum concentration of protein was obtained with 2 Hz, while maximal bicarbonate output occurred at 12 Hz.

In 10 pigs 3–4 frequencies were used in the same pig. In 3 pigs 6–7 different frequencies were studied. In both cases 5 min periods of stimulation and intervening intervals of 10 min were used. The individual values for flow, protein output and bicarbonate output expressed as per cent of the maximum values obtained, are shown in Fig. 1. Although differing in sensitivity, frequency-dependent responses were clearly demonstrated in each pig.

TABLE IV. Potentiation by exogenous secretin of the pancreatic exocrine response to vagal stimulation

Number of expts.	Flow of juice %	Protein conc. %	Bicarbonate conc.	Protein output %	Bicarbonate output
13	184 (133–312)	48 (21–80)*	111 (103–126)	89 (39–201)	214 (164–315)

Submaximal or maximal vagal stimulation (5 min) was carried out 30 and 60 min after injection of secretin 0.5 C.U./kg. The data are presented as the response at 30 min in per cent of response to the stimulation at 60 min, with each animal being its own control, and expressed as the median followed by the 95% confidence limits. Because the basal rate of secretion (60 min after secretin) as well as the secretin-stimulated secretion (30 min after secretin, Table I) constituted less than 5% of the total response to vagal stimulation, these values have not been subtracted.

* Significant changes.

TABLE V Potentiation by acidification of the duodenal bulb of the pancreatic exocrine response to vagal stimulation.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Perfusion at pH 4					
0–10 min	0.15 (0–0.48)	—	—	—	—
10–20 min	0.03 (0–0.06)	—	—	—	—
4 Hz Vagal stimulation 20–25 min	18.1 (13.2–30.0)	55 (38–184)	108 (70–122)	1.17 (0.5–4.24)	1.90 (0.38–3.64)
Perfusion at pH 1					
0–10 min	0.51 (0–1.2)	—	—	—	—
10–20 min	0.5 (0.6–4.0)*	—	—	—	—
4 Hz Vagal stimulation 20–25 min	42 (23.2–57.5)	28 (24–34)	123 (48–145)	1.18 (0.63–3.10)	5.56 (2.47–8.26)
Response to vagal stimulation at pH 1 in per cent of the response at pH 4	225 (192–467)	61 (16–80)*	127 (89–140)*	149 (39–331)*	281 (183–633)*

The duodenal bulb was perfused with buffers of pH 4 and pH 1 for 25 min in 4 pigs. After 20 min vagal stimulation was performed for 5 min at 4 Hz. Results are presented as the median and the total range.
* Significant changes.

d) *Potentiation by secretin of the response to vagal stimulation*

1) In 13 pigs submaximal or maximal vagal stimulations were carried out 30 min after secretin administration, and repeated again after another 30 min. Thirty min after the secretin injection, the plasma concentration of secretin was approximately twice as high as the basal concentration (see below) and the pancreatic flow of juice slightly increased (Table I). At 60 min both parameters had reached basal levels.

If the fluid and bicarbonate response to vagal stimulation was merely added to the secretory response to secretin 30 min after the injection, one would have expected a combined

TABLE VI The effect of splanchnicotomy on the pancreatic exocrine response to vagal stimulation.

	Flow of juice	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output %
Vagal stimulation 8–20 Hz and bilateral splanchnicotomy 5. Median (range)	123 (79–349)	103 (75–124)	110 (88–170)	144 (84–381)	125 (70–609)

Per cent of the response to vagal stimulation before splanchnicotomy with each animal being its own control

TABLE VII. Pancreatic exocrine response to electrical stimulation of the left splanchnic nerve.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc mmol/l	Protein output g/h	Bicarbonate output mmol/h
Secretin 0.5 C.U./kg. Second 5 min period (6)[a]	51 6 (36–60)	0.4 (0.2 1.9)	140 (132 156)	0.02 (0.007–0.09)	7.0 (4.9–9
Vagal stimulation at 8–20 Hz. First 5 min (6)	33 9 (22.2–48 0)	114 (85–167)	131 (1 4–138)	3.8 (2.2–6.9)	4.3 (3 0–4
Splanchnic stimulation at 10–20 Hz (10)	0.30 (0.0–1 80)				

Pancreatic secretion during splanchnic stimulation was investigated in 10 pigs, in 6 of these stimulation was also performed, and 6 received secretin 0.5 C.U./kg. The data are presented as the med followed by the total range

response which exceeded the response 60 min after secretin (flow 31 ml/h, Table II) by 2.1 ml/h (Table I) *i.e.* 7%. However we found fluid and bicarbonate responses which constituted 184 and 214% of the responses without secretin (Table IV), which indicates that slightly elevated secretin concentrations markedly potentiate the fluid and bicarbonate response to vagal stimulation. The protein concentration was reduced to 48%, but the output was unchanged (89%)

2) In 4 pigs the vagi were stimulated with low frequency (4 Hz) during simultaneous perfusion of the duodenal bulb with isotonic buffers of pH 4 and 1 respectively but similar titratable acidity The perfusion of the duodenal bulb at pH 4 did not affect pancreatic secretion, while a stimulated flow of juice was noted in all pigs during perfusion at pH 1. The volume and bicarbonate response to vagal stimulation under these circumstances was again markedly potentiated as shown in Table V

e) *The splanchnic nerves*

The results of vagal stimulation before and after sectioning of the splanchnic nerves are shown in Table VI In 4 of 5 pigs flow protein and bicarbonate output were enhanced after splanchnicotomy ($p = 0.075$ U-test)

TABLE VIII. Pancreatic exocrine response to simultaneous splanchnic and vagal stimulation

	Flow of juice %	Protein conc. %	Bicarbonate conc. %	Protein output %	Bicarbonate output %
Vagal stimulation, 8 Hz, splanchnic stimulation 10 Hz (n = 5). Median (range)	7 6 (4–37.5)*	85.7 (67 137)	90 (42–98)	10.5 (3–49 7)	5.6 (4–33.8)

Per cent of the response to vagal stimulation alone with each animal being its own control.
Significant changes.

TABLE IX. Pancreatic exocrine response to prolonged vagal stimulation.

Interval of collection (in min)	Flow of juice	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output
0–5	100	100	100	100	100
5–10	97 (70–111)	77 (47–127)	101 (100–110)	70 (15–127)	100 (71–111)
10–15	89 (50–106)	63 (30–121)	102 (93–110)	80 (47–103)	89 (49–116)
15–20	82 (77–100)	64 (45–106)	103 (74–108)	50 (36–86)	81 (74–89)
20–25	93 (64–113)	69 (35–103)	106 (97–121)	44 (32–96)	96 (68–119)
25–30	133 (56–153)	49 (37–79)	103 (94–126)	66 (23–97)	141 (53–160)

Vagal stimulation (8 Hz) was continued for 30 min in 6 pigs. Juice was collected in 5-min periods. The values represent per cent of the response during the first 5 min, with each pig being its own control (median and total range).

Splanchnic nerve stimulation was performed in 10 pigs, which showed similar responses to secretin and vagal stimulation as the others (Table VII). The pancreatic exocrine secretion did not increase above basal levels. It was, however, not possible to determine if splanchnic stimulation inhibited the basal secretion. The efficiency of the splanchnic stimulation was evidenced by a pronounced rise in arterial blood pressure a few seconds after the start of stimulation. In 5 pigs the response to simultaneous submaximal vagal stimulation (8 Hz) and splanchnic stimulation was examined. The results which appear in Table VIII are presented as the response to combined stimulation in per cent of the response in the same pig to vagal stimulation at the same frequency alone. The protein concentration remained unchanged but the other 4 parameters of secretion were inhibited, the greatest reduction being for flow of juice.

f) Reproducibility of responses

Tables IX and X show the results of prolonged (30 min) and repeated vagal stimulation. It can be seen, that an increase in exocrine secretion lasted as long as stimulation was continued, and that similar responses were elicited by repeated stimulations.

g) Pharmacological analysis

Table XI shows the results of vagal stimulation at various frequencies after various doses of atropine. It is obvious that atropine does not inhibit the vagally stimulated flow of juice, the bicarbonate concentration or the bicarbonate output. The protein concentration how-

TABLE X. Pancreatic exocrine response to vagal stimulation. Reproducibility (9 expts. in 7 pigs).

	Flow of juice[a]	Protein conc.	Bicarbonate conc.	Protein output[a]	Bicarbonate[a] output %
Median (range)	100 (68–120)	96 (70–147)	107 (96–129)	105 (61–115)	108 (73–155)

Per cent of response to first stimulation. The stimulations were repeated after 0.5–3 h. Median and total range.

TABLE VII Pancreatic exocrine response to electrical stimulation of the left splanchnic nerve.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc mmol/l	Protein output g/h	Bicarbonate output mmol/h
Secretin 0.5 C.U./kg. Second 5 min period (6)[a]	51 6 (36–60)	0.4 (0.2 1 9)	140 (132 156)	0.02 (0 007–0.09)	7.0 (4.9-9 6)
Vagal stimulation at 8–20 Hz. First 5 min (6)	33 9 (22.2–48 0)	114 (85–167)	131 (124–138)	3.8 (2.2–6.9)	4.3 (3.0-6 0)
Splanchnic stimulation at 10–20 Hz (10)	0.30 (0 0–1 80)				

Pancreatic secretion during splanchnic stimulation was investigated i 10 pigs, i 6 of these a vagal stimulation was also performed, and 6 received secreti 0.5 C.U./kg. The data are presented as the median followed by the total range

response which exceeded the response 60 min after secretin (flow 31 ml/h, Table II) by 2.1 ml/h (Table I) *i.e* 7%. However we found fluid and bicarbonate responses which constituted 184 and 214% of the responses without secretin (Table IV), which indicates that slightly elevated secretin concentrations markedly potentiate the fluid and bicarbonate response to vagal stimulation The protein concentration was reduced to 48 %, but the output was unchanged (89%)

2) In 4 pigs the vagi were stimulated with low frequency (4 Hz) during simultaneous perfusion of the duodenal bulb with isotonic buffers of pH 4 and 1 respectively but similar titratable acidity The perfusion of the duodenal bulb at pH 4 did not affect pancreatic secretion, while a stimulated flow of juice was noted in all pigs during perfusion at pH 1 The volume and bicarbonate response to vagal stimulation under these circumstances was again markedly potentiated as shown in Table V

c) *The splanchnic nerves*

The results of vagal stimulation before and after sectioning of the splanchnic nerves are shown in Table VI In 4 of 5 pigs flow protein and bicarbonate output were enhanced after splanchnicotomy ($p = 0.075$ U-test)

TABLE VIII Pancreatic exocrine response to simultaneous splanchnic and vagal stimulation.

	Flow of juice %	Protein conc. %	Bicarbonate conc. %	Protein output %	Bicarbonate output
Vagal stimulation, 8 Hz, splanchnic stimulation 10 Hz (n = 5). Median (range)	7 6 (4–37.5)	85 7 (67 137)	90 (42–98)	10.5 (3–49 7)	5 6 (4–33.8)*

Per cent of the response to vagal stimulation alone with each animal being its own control.
Significant changes.

TABLE XIII. Pancreatic exocrine secretion after acetylcholine infused intraarterially for 5 min.

Rate of infusion	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/l
	1.2	—	—	—	—
0.010–0.015 mg/min	4.8	110	95	0.52	0.53
	9.0	243	110	2.18	0.81
0.04–0.05 mg/min (5)	10.2 (2.4–12.9)	75 (41–168)	98 (90–120)	0.66 (0.10–1.81)	0.99 (0.36–1.18)
	8.6	137	110	1.18	0.95
0.12–0.15 mg/min	3.6	51	145	0.18	0.52
0.4–0.5 mg/min (7)	11.4 (5.8–34.8)	134 (64–153)	96 (81–118)	1.43 (0.36–5.16)	0.98 (0.64–4.1)
	6.7	120	86	0.81	0.57
mg/min	4.8	236	90	1.13	0.43
	2.2	—	—	—	—
0 mg/min	4.8	132	95	0.63	0.45
	12.0	222	104	2.66	1.25
	0.3	—	—	—	—
00 mg/min	3.6	237	105	0.85	0.38

The data presented as individual values or median followed by total range.

ponse after ACh vs. secretin 36.7% (median range 6.9–75) flow ACh vs. vagal stimulation 9.5 (9.0–75), protein concentration, ACh vs. vagus 180% (127–259) bicarbonate concentration, ACh vs. vagus 75% (64–87).

In six pigs acetylcholine was infused in doses of 0.1–0.5 mg/min after the administration of atropine, 0.5 mg/kg. In no case was the exocrine secretion stimulated above basal levels. In four pigs ACh 0.5 mg/min was infused after the administration of hexamethonium. The results are shown in Table XIV Hexamethonium stimulated rather than inhibited the exocrine response.

In four pigs the exocrine response to vagal stimulation was investigated after beta-adrenergic blockade. In each case beta-blockade was evidenced by a slight lowering of blood pressure and a decrease in pulse rate. The results are shown in Table XV The flow and protein

TABLE XIV. Pancreatic exocrine response to intraarterial acetylcholine after pharmacological blockade.

Dose of acetylcholine and blockade		Flow of juice ml/h	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output
0.1–0.5 mg/min 6, atropine 0.5 mg/kg		0.90 (0.0–2.2)	—	—	—	—
0.5 mg/min Hexamethonium 10–30 mg/kg	4	170 (32–258)*	144 (103–163)*	97 (82–118)*	239 (103–375)*	169 (67–306)*

* Per cent of the response to the same dose of acetylcholine without blockade, with each animal being its own control. The data are presented as the median and total range.

TABLE XI Pancreatic exocrine response to vagal stimulation after atropine

Frequency and dose of atropine	Flow of juice %	Protein conc.	Bicarbonate conc. %	Protein output[a] %	Bicarbonate output %
2 Hz, mg/kg (6)	113 (52–233)	10 4 (4 9–37)*	132 (62 143)	12.9 (3.6–79)	137 (32 339)
8 Hz, 2 mg/kg (6)	111 (68–288)	5.5 (1.8–11.3)	115 (102 132)	6.3 (1 4–21.8)*	123 (69–340)
20 Hz, 2 mg/kg (12)	92 (71 144)	15 6 (7 7 23.2)*	100 (95–111)	18.2 (12.6–39 6)*	89 (65–279)
2 20 Hz, 0.5–2 mg/kg (25)	104 (76–144)	10.0 (6.9–14 0)	111 (102 117)	12.3 (9.0–18.0)*	111 (77–152)

Per cent of response without atropine (Table III) with each animal being its own control. The data are expressed as the median followed by the total range (2 and 8 H) or median followed by 95% limits of confidence (20 and 2 20 Hz) of the median.

Significant changes.

ever and hence the protein output was markedly decreased in all experiments. In two pigs atropine was administered in high doses as a primed infusion (0.5 mg/kg and 0.5 mg/kg h) but also with this technique it was impossible to inhibit the exocrine response to vagal stimulation The results of an atropine dose-response study are shown in Table XII The decrease in protein concentration and output was dependent on the dose of atropine, whereas the flow and bicarbonate secretion remained unaffected

Hexamethonium, on the other hand, completely abolished the response to vagal stimulation. In eight animals which all responded satisfactorily to secretin (flow 51 6 (36.0-60 8 ml/h) and vagal stimulation (flow· 30 (22.2–66.0) ml/h) there was no detectable deviation from basal secretion during vagal stimulation after hexamethonium blockade (flow· 0 2 (0 0–0 6) ml/h)

We then studied the exocrine response to intraarterially infused acetylcholine. The results are summarized in Table XIII The pancreatic exocrine response to acetylcholine was dose dependent

A stimulated flow was noted with 10 μg/min while maximum response required 0.5 mg/min Higher doses were less effective. The high doses (> 10 mg/min) severely depressed cardiorespiratory functions of the animals. The juice was characterized by its high protein concentration (134 1 g/l) and a variable concentration of bicarbonate. When compared to the responses to maximal secretin and vagal stimulation, with each animal being its own control the following results of stimulation with 0.5 mg/min ACh were obtained flow of

TABLE XII Pancreatic exocrine response to vagal stimulation after various doses of atropine

Frequency and dose of atropine	Flow of juice %	Protein conc. %	Bicarbonate conc.	Prot in output[a]	Bicarbonate output %
8 Hz	100	100	100	100	100
8 Hz+0 005 mg/kg	150	59	120	89 1	180
8 Hz+0 05 mg/kg	150	8 7	129	13 1	193
8 Hz+0.5 mg/kg	133	4 9	132.0	6.5	176

Per cent of response without atropine. Stimulation for 5 min

XVII. Effect of vagal stimulation on the secretin concentration in portal venous plasma during acidification of the duodenal bulb.

	Minutes after start of stimulation									
	15	10	5	0	0.5	1	3	5	10	15
...tin conc. pmol/l ...an and total range).	2.1 (0–6.4)	3.5 (1.7–7.6)	2.8 (0.1–13.5)	4.5 (1.4–8.4)	4.4 (1.4–7.4)	4.0 (1.4–7.0)	2.9 (1.4–6.0)	4.6 (1.3–7.7)	4.0 (1.6–9.6)	3.3 (2.0–10.3)

...stimulation was carried out at 4 Hz in 4 pigs during perfusion of the duodenal bulb with buffer of 0 and buffer of pH 1.0. The differences in the plasma concentration of secretin during pH 1 and are given in the Table, with each animal being its own control. The duodenal bulb was perfused from to 5 min after the start of stimulation.

...e concentration of secretin in portal plasma during perfusion of the duodenal bulb citrate buffer at pH 4 did not differ from the concentration in non-perfused animals, vagal stimulation did not change this concentration. Perfusion of the duodenal bulb citrate buffer at pH 1 caused a significant increase in the portal secretin concentration ...le XVII). Again, vagal stimulation did not influence the concentration. The results are ...ented as the difference between the concentrations of secretin obtained during perfusion ...H 4 and pH 1 with each animal being its own control.

Discussion

...results presented above A) confirm and extend earlier observations on the importance ...he vagal innervation in the control of pancreatic exocrine secretion (Harper 1972, Hick... 1970 b, Thomas 1967 Preshaw 1967 Sjödin 1977), B) provide evidence, that the sympa...tic nervous system may modulate the secretory response to vagal stimulation, C) suggest ...t vagal potentiation of humorally regulated secretion is of great physiological importance ...l D) raise the question of the mechanism involved in the secretory response to vagal ...mulation.

...the dog and the cat (Hickson 1970 b, Brown *et al.* 1967 Lenninger and Ohlin 1971) ...gal stimulation enhances enzyme output but causes only a sparse flow of juice. It was ...erefore of considerable interest, when Hickson reported that in the pig vagal stimulation, ...art from increasing protein secretion, causes a profuse flow of juice with a high content of ...carbonate (Hickson 1963, 1970 b). Circumstantial evidence has been presented that other ...ecies, including man (Comline *et al.* 1964 Thomas 1967) may respond similarly. Our ...dings confirm the potency of vagal stimulation on the secretion of the porcine pancreas. ...he flow and bicarbonate responses amounted to 59 and 55% of the maximal secretory ...sponse to secretin. The response was clearly frequency dependent with a half-maximal ...fective frequency of 2–4 Hz and a maximal effective frequency of 12 Hz (Table III). In 3 ...f 4 expts. an increased flow of juice was observed with frequencies below 1 Hz. This is in

TABLE XV Pancreatic exocrine response to vagal stimulation after adrenergic blockade

	Flow of juice	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output
Vagal stimulation 8–12 Hz + Propranolol 1 mg/kg, n = 4 Median (range)	125 (70–153)	163 (112 349)	89 (77 109)	194 (140–332)	111 (51–144)
Vagal stimulation 8 12 Hz + Propranolol as above + Phenoxybenzamine 1 mg/kg, n = 5 Median (range)	142 (64–315)	101 (63–248)	100 (72 105)	143 (63–401)	144 (64–331)

Per cent of response to vagal stimulation without blockade with each animal being its own control

response were increased (p = 0 17) whereas the bicarbonate response was not changed significantly

Finally in 5 pigs the response to vagal stimulation was investigated after a combined alpha and beta-adrenergic blockade. In these experiments, all fluid losses were replaced with Macrodex 6 % in saline (Pharmacia, Uppsala, Sweden) to avoid excessive hypotension Again the response to vagal stimulation was enhanced, when compared to stimulation before blockade (p = 0 075 (Table XV)

Secretin determinations

Blood samples for secretin determination were taken during vagal stimulation in 25 experiments in 17 pigs (Table XVI). Although there was no significant change in the secretin concentration in portal plasma at any time, the median value decreased by 15 immediately after the start of vagal stimulation The arterial concentration of secretin (measured in two pigs), remained unchanged during stimulation

In 10 pigs blood samples for secretin determination were obtained 30 min after the administration of secretin 0 5 Clinical Units kg⁻ The median concentration was 5.3 (2.7–8.6) pmol/l significantly and approximately 2 times greater than the basal levels.

TABLE XVI Effect of vagal stimulation on the secretin concentration in portal venous plasma.

	Minutes after start of stimulation									
	−15	10	5	0	0.5	1	3	5	10	15
Plasma secretin pmol/l 25 expts. in 17 animals. Median + 95 % confidence limits of the median	2.7 (1 6–4.0)	2.1 (1.3–3 1)	2.0 (1.5–2.4)	2.6 (1 7 3.0)	2.2 (1.8–3.3)	2.4 (1.5–2.8)	2.6 (1.8–2.9)	2.6 (2.4–3 4)	2.6 (1 8–3.4)	2.5 (1.8–3.3)

Frequency of stimulation was 8–20 Hz in 13 expts. and 4 H in the remaining 12. Since the stimulation did not significantly influence the plasma secretin concentration in either group, the groups were combined in the Table.

ble XVII. Effect of vagal stimulation on the secretin concentration in portal venous plasma during acidification of the duodenal bulb.

	Minutes after start of stimulation									
	15	10	5	0	0.5	1	3	5	10	15
Secretin conc. pmol/l (median and total range), 4	2 1 (0–6 4)	5.5 (1 7–7 6)	2 8 (0.1–11.5)	4.5 (1.4–8.4)	4 4 (1.4–7 4)	4 0 (1 4–7.0)	2.9 (1 4–6 0)	4 6 (1.3–7 7)	4 0 (1.6–9 6)	3.3 (2.0–10 3)

gal stimulation was carried out at 4 Hz in 4 pigs during perfusion of the duodenal bulb ith buffer of 1 4 0 and buffer of pH 1 0. The differences in the plasma concentration of secretin during pH 1 and I 4 are given in the Table, with each animal being its own control. The duodenal bulb was perfused from 20 to 5 min after the start of stimulation.

The concentration of secretin in portal plasma during perfusion of the duodenal bulb ith citrate buffer at pH 4 did not differ from the concentration in non-perfused animals, id agal stimulation did not change this concentration. Perfusion of the duodenal bulb th citrate buffer at pH 1 caused a significant increase in the portal secretin concentration Table XVII). Again, vagal stimulation did not influence the concentration. The results are esented as the difference between the concentrations of secretin obtained during perfusion pH 4 and pH 1 with each animal being its own control.

Discussion

he results presented above A) confirm and extend earlier observations on the importance f the vagal innervation in the control of pancreatic exocrine secretion (Harper 1972, Hickson 1970 b, Thomas 1967 Preshaw 1967 Sjodin 1977), B) provide evidence, that the sympathetic nervous system may modulate the secretory response to vagal stimulation, C) suggest that vagal potentiation of humorally regulated secretion is of great physiological importance nd D) raise the question of the mechanism involved in the secretory response to vagal stimulation.

the dog and the cat (Hickson 1970 b, Brown *et al.* 1967 Lenninger and Ohlin 1971) agal stimulation enhances enzyme output but causes only sparse flow of juice. It was herefore of considerable interest, when Hickson reported that in the pig vagal stimulation, apart from increasing protein secretion, causes a profuse flow of juice with a high content of icarbonate (Hickson 1963 1970 b). Circumstantial evidence has been presented that other pecies, including man (Comline *et al.* 1964, Thomas 1967) may respond similarly Our indings confirm the potency of vagal stimulation on the secretion of the porcine pancreas. The flow and bicarbonate responses amounted to 59 and 55% of the maximal secretory esponse to secretin. The response was clearly frequency dependent with a half-maximal effective frequency of 2–4 Hz and a maximal effective frequency of 12 Hz (Table III) in 3 of 4 expts. an increased flow of juice was observed with frequencies below 1 Hz. This is in

agreement with the frequency response pattern of other gastrointestinal functions k be controlled by parasympathetic innervation (Hillarp 1960) The response to vagal stim tion was highly reproducible (Table X), and a stimulated rate of secretion could be m tained as long as the electrical stimulation was continued (Table IX).

Compared to the measured basal flow of juice the response to maximal vagal stimul represent a 55-fold increase. The basal flow is comparable to that obtained in the pancreas from pigs of similar size, when perfused with a completely synthetic me (Lindkær Jensen *et al* (in press))

B

Splanchnic stimulation strongly inhibited the response to simultaneous vagal stimul mostly through an inhibition of the flow of juice (Table VIII) Furthermore, the to vagal stimulation was enhanced after cutting the splanchnic nerves. Likewise, aft and/or β-adrenergic blockade most animals showed increased responses. Thus, while noth can be deduced about the mechanism of the sympathetic inhibitory effect the results sugg that the sympathoadrenal system may significantly modulate the pancreatic exocrine se tion in response to vagal activity

This finding also implies that attempts to elicit vagally induced fluid and bicarbon secretion by means of central glucopenia (insulin and 2-deoxy-glucose) are likely to because of the simultaneous activation of the sympathetic nervous system.

Stimulatory effects of splanchnic stimulation (Thomas 1967) could not be demonstrat

C

Our finding of potentiation by secretin of the response to vagal stimulation is in agreem with previous findings in pigs (Magee and White 1965 Hickson 1970 a) and cat (Bro *et al* 1967) The recent development of sensitive and accurate radioimmunoassays secretin (Schaffalitzky de Muckadell and Fahrenkrug 1977 c) enabled us to ascertain this phenomenon was studied during elevations of the plasma level of secretin which comparable to those seen in physiological circumstances. The levels attained (5.3 and pmol/l respectively) were well within the range found during and after ingestion of a m (Schaffalitzky de Muckadell and Fahrenkrug (in press) Fahrenkrug and Schaffalitzky Muckadell 1977) The pancreatic fluid and bicarbonate responses to vagal stimulation und these circumstances amounted to 184 and 214% (exogenous secretin) and to 225 and 2 (duodenal acidification) of the responses to vagal stimulation in control experiments in same animals without elevations of the plasma concentration of secretin These res have important physiological implications. It is reasonable to believe that the duod bulb (which was the only part of the duodenum perfused in these studies) is rather c stantly exposed to fluid of low pH (close to 1) in contrast to the rest of the duodenum, wh only occasional "spikes of pH lower than 4–5 are found in the fasting state as wel during the ingestion of a meal (Schaffalitzky de Muckadell and Fahrenkrug (in pres Our results prove, that acidification of the bulb alone is sufficient to stimulate the secret of secretin and pancreatic secretion of fluid and bicarbonate, but the response is sm But if one adds to this a moderate vagal activity a greatly increased pancreatic secreti

tissues, which is sufficient to explain a considerable part of the pancreatic secretion believed to take place after a meal.

)

The pancreatic secretion of protein was found to be markedly enhanced by vagal stimulation in agreement with previous investigations (Harper 1972, Thomas 1967 Preshaw 1967). The response was strongly inhibited by low doses of atropine, completely abolished by hexamethonium, mimicked closely by acetylcholine, the effects of which were also abolished by atropine but unaffected by hexamethonium. It is therefore reasonable to believe, that the vagal stimulation of protein secretion follows the classical pathways of the parasympathetic nervous system, *i.e.* we stimulate preganglionic fibres, the activity of which is transmitted via cholinoceptive nicotinic receptors in autonomic ganglia to postganglionic fibres, which through the release of acetylcholine near cholinoceptive muscarinic receptors triggers the target organ to secrete. The stimulated secretion of fluid and bicarbonate behaves differently however the most important difference being the complete atropine-resistance of the response to vagal stimulation. Also these findings are in agreement with previous investigations (Hickson 1970 b, Brown *et al* 1967 Lenninger and Ohlin 1971). Atropine resistance of effects mediated by parasympathetic nerves is known to occur in many systems of the organism (Innes and Nickerson 1975 Ambache 1955). The phenomenon was discussed in detail by Ambache (1955), who offered four explanations to this effect. Firstly the theory of Dale and Gaddum (1930) which implied the existence of specialized *neuro-effector junctions*, in which an essentially "muscarine-like" action of the acetylcholine released by nerve impulses was locally protected from the effects of atropine (the "proximity theory"). We find it difficult to accept this concept of atropine-protected choline receptors since atropine effectively blocked the stimulatory action of intraarterially administered acetylcholine.

It is also difficult to understand that cholinoreceptors for fluid and bicarbonate secretion should differ so much in this respect from the receptors for protein secretion, which apparently are readily accessible for atropine.

Secondly atropine resistance, which is due to destruction of atropine in the animal, should be considered, since a genetically determined "atropine-esterase" (atropine acyl hydrolase) activity is known to occur in rabbits (Goldstein *et al.* 1969). The negative results of continuous-infusion experiments, and the fact that the pancreatic protein secretion was strongly inhibited by low doses of atropine refutes this possibility. Similarly acute tolerance or "tachyphylaxis" would be a highly conjectural solution to the problem, since we never saw any inhibition, regardless of the time, dose, or number of injections given. Thirdly an atropine resistant secondary formation of a pharmacological agent *e.g.* bradykinin, was considered. To apply such a mechanism, however is but to refer the problem to a higher level of complexity

Finally atropine resistance may be due to non-cholinergic transmission.

The response is not dependent on the availability of or the secretion rate of secretin, since it could be elicited in pigs after surgical removal of all tissue known to produce secretin (Hickson 1970 b) and since the secretin concentration in portal venous plasma was unaffected by vagal stimulation. The portal vein was selected for sampling to ensure the highest

degree of sensitivity but the results of secretin determination in arterial plasma confir that the splanchnic production of secretin was unchanged by vagal stimulation. The ini lowering of the portal vein concentration of secretin is probably a consequence of the sti stimulating effect on portal flow elicited by vagal stimulation (Fahrenkrug *et al* 1978).

Other gastrointestinal hormones (gastrins, cholecystokinin) may be liberated during va stimulation, and may be responsible for part of the pancreatic exocrine response in th intact animal but these hormones could not explain the secretory response to vagal stimula tions after removal of the stomach and the small intestine (Hickson 1970 b). Furthermor gastrins and cholecystokinin are known to stimulate strongly the pancreatic secretion o enzymes and are therefore less likely to participate in the secretory response to vagal stimula tion in our experiments since the protein response was easily inhibited by atropine.

The abdominal vagus is known to contain adrenergic fibres (Liedberg *et al* 1973). A adrenergic contribution to the vagally stimulated pancreatic secretion is less likely i view of the lack of inhibitory effect of adrenergic blockade.

The results therefore indicate that acetyl choline may not be the final transmitter o stimulation to the pancreatic secretion of fluid and bicarbonate. Instead we suggest th the transmitter function is exerted by Vasoactive Intestinal Polypeptide (VIP) (Said 197 VIP a 28 amino acid peptide with a close similarity to secretin in structure and biologi effects (Said 1975 Bodanszky *et al* 1933) has been localized to neurons in many parts o the body (Larsson *et al* 1976 a, b, Said and Rosenberg 1976, Bryant *et al* 1976, Sundl *et al* 1977 Larsson *et al* 1977 a, b) and VIP-containing nerves have been shown to innerva acini and ganglia of the porcine pancreas (Larsson *et al* 1978) By combined immunochem istry immunocytochemistry and electron microscopy VIP has been localized to synapt vesicles of VIP-containing nerves (Larsson 1977 Giachetti *et al* 1977 Emson *et al*. 197 A release of VIP from nervous tissue has been elicited by the aid of potassium (Giache *et al* 1977 Emson *et al* 1978) Furthermore, electrical stimulation of the vagus produc an atropine resistant splanchnic release of VIP in anesthetized pigs (Fahrenkrug *et al* 197 Schaffalitzky de Muckadell *et al* 1977 b) and during stimulation an increased release o VIP from the pancreas can be demonstrated (unpublished observations).

We admit that the only reason to imply that acetylcholine is not the transmitter of th vagal effects on the pancreas is the fact that the fluid and bicarbonate secretion followi vagal stimulation is atropine resistant. To imply that VIP is the transmitter we will have t explain in addition the effect of acetylcholine on the fluid and bicarbonate secretion (Tab XIII) which is observable also in the totally isolated pancreas, perfused with a synthet medium at constant flow (Holst, Lindkær Schaffalitzky de Muckadell and Fahrenkru unpublished). Two explanations present themselves either the acinar cells are equippe with muscarinic receptors, which are not innervated, and therefore do not participate i the vagally induced secretion, or the VIP-nerves may contain such receptors. That isolate acinar cells possess muscarinic receptors has been amply documented (Christophe *et a* 1976) and the stimulated release of VIP after acetylcholine (Fahrenkrug *et al* 1978) suppo the idea of muscarinic receptors on the VIP nerves. A combination of both might well b the real explanation.

The development of sensitive and accurate radioimmunoassays for secretin has mad

ssible a delineation of the contribution of secretin in the control of pancreatic secretion of id and bicarbonate. It appears that there is ample space for other control systems as well lanos *et al.* 1977). Among these, the peptidergic nervous systems may very well turn out be of primary importance.

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Methods

ecordings from left and right sides of the CT were obtained in Sprague-Dawley rats weighing between 0 and 450 g. Before the recording the CT on one side was interrupted in the middle ear. This was generally done either by injecting 0.2–0.4 ml of 99.5 % alcohol through the tympanic membrane under light Hypnorm® algesia or by severing the CT in the middle ear. In some animals colchicine, 5 mg/ml, or Marcain 5 mg/ml, a long lasting local anesthetic, was injected at the same site. Marcain was administered every 6 h as used. The animals were then left for time periods ranging from 1 h to 3 days. After this period the nimals were anaesthetized with Mebumal and Hypnorm. The CT was dissected, cut and put on recording electrodes, the tongue was then stimulated with solutions of 0.3 or 0.1 M NaCl, 0.02 M quinine hydrochloride, 0.02 or 0.03 citric acid and 0.3 M sucrose. The stimuli were applied to the tongue for about 3 s, ...ed with water rinses for about 30 s.

The nerve activity was recorded as integrated trace and the average magnitude of 10 integrated recordings to a particular taste stimulus at each time interval was determined and used as measure of the taste response.

In 5 rats the right facial nerve was cut between the geniculate ganglion and the CNS 3–4 weeks before recording experiment. This was done to cause the selective degeneration of the efferent fibres in the CT nerve. The technique has been described elsewhere (Farbman and Hellekant 1978).

Results

Recordings from normal rats

Since it is possible, though unlikely that there is a systematic difference between the responses of the right and left CT the response of nerves from the two sides were compared in 12 rats. The result is shown in Fig. 1 in which the amplitudes of the responses of the right and left CT to all taste stimuli used have been plotted. The regression line for these points was calculated (y = 0.82x + 1.7) and drawn in Fig. 1. The correlation coefficient between the responses of left and right CT is 0.93. Fig. 1 may indicate a difference between the two sides. However if there was a difference between right and left CT this was not significant. Further as can be seen from Fig. 1 there was a rather large variation in amplitude of the responses from animal to animal. There was no difference in the shape of the responses from right and left CT.

Recordings from denervated rats after injection of alcohol

Fig. 2 shows data from 85 rats. The magnitude of the responses to 0.3 ml NaCl has been plotted against the time after the CT was interrupted in the middle ear. Each animal was tested only at one time interval. It is evident that the magnitude of the response decreased with time after destruction.

After about 5 h, the response in the nerve had declined to about 50 %. No response at all could be recorded after 15 h in any of the 12 animals studied at this time interval.

As a comparison the average response magnitude of the CT of the other side has been included—the horizontal line.

In addition to the decline in response with time there is an indication of a stage of increased responsiveness during the first hours after the nerve has been cut. There is a strong correlation between the response magnitude at each interval and postoperative time (r = 0.86) which is best described by the equation

$$y = 77 - 28 \ln x$$

Fig. 2 is based on data from animals in which injections of alcohol in the middle ear were used to interrupt the CT. Since it might be thought that diffusion of alcohol along the

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Decline and disappearance of taste response after interruption of the chorda tympani proper nerve of the rat

By

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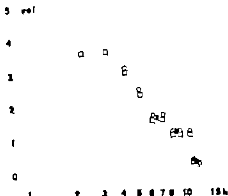
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Abstract

HELLEKANT G V GOPAL and Y NINOMIYA. *Decline and disappearance of taste resp[onse] after interruption of the rat chorda tympani proper nerve* Acta physiol scand. 19[79] *105* 52 57

The response amplitude in the chorda tympani proper nerve of rats to taste stimulation has been studied [at] different times afte interruption of the nerve n the middle ear The results show that the response [of the] nerve declined and disappeared completely within 15 h after the nerve was interrupted. As a first sign [of] deteriorating function at go of hypersensitivity was observed. Results obtained during application [of] local anesthetic or colchicine and section at different distances from the tongue indicate that the decrea[se in] response was the result of n interruption of axoplasmic flow from the nerve cell bodies in the genicul[ate] ganglion to the taste buds.

The fact that the morphological integrity of gustatory structures depends on intact gustat[ory] nerves is well documented (*cf* Kennedy 1972) In the rat, studies have shown the disappe[ar]ance of taste buds after section of gustatory sensory nerves such as the glossopharyng[eal] (Guth 1957) or chordalingual (Whiteside 1927) and regeneration of taste buds after [re]innervation (Guth 1958 Oakley 1967 Zalewski 1969) Recent electron microscope stud[ies] of these structures have not only confirmed this but have also demonstrated the sequ[ence] of degenerative changes at the cellular level Thus in the study by Farbman (1969) the fi[rst] morphological changes in extra and intra gemmal nerve endings were observed in so[me] specimens as early as 3–6 h after the combined chorda-lingual nerve had been cut. Mo[re] consistent degenerative changes in these structures were observed between 12 and 24 [h]. Such morphological changes may be reflected in changes of function of these structures [as] assessed by electrical recordings during an acute experiment This paper describes chan[ges] of function in the peripheral sense of taste after section of the chorda tympani pro[per] nerve (CT) in rat.



g. 3 The diagram shows the decline and disappearance of the CT response in one animal during peated stimulation with 0.03 m citric acid (□) and 1 m NaCl (○). During the recording period the rve was kept in mineral oil. On four occasions the oil was removed and the nerve rinsed with Ringer solution (R) which was then replaced with mineral oil.

hough the response of the other side has disappeared. From this it can be concluded that the presence of anesthesia did not change the events described.

Recordings from denervated rats after injection of colchicine or local anesthetic

In addition to conducting impulses, gustatory nerves may transport substances which are vital for the maintenance of taste buds. Local anesthetic and colchicine can be used experimentally to differentiate between impulse propagation and axonal transport. The local anesthetic blocks impulses but leaves axoplasmic transport relatively unaffected (Byers *et al.* 1973), while colchicine allows the passage of impulses (Cuenod *et al.* 1974) but blocks the transport mechanism. Thus in 6 rats a longlasting local anesthetic was injected every sixth hour into the ear instead of alcohol. The results are illustrated by the squares in Fig. 2. Two conclusions can be drawn. A response could be recorded for a much longer time than when alcohol was used. But the anesthetic did not leave the response unaffected which is corroborative with the observations of Byers *et al.* (1973). Colchicine, on the other hand, caused a decline and disappearance in the same way as if the nerve had been interrupted by alcohol (not shown in Fig. 2). From this it seems that the mere interruption of impulse transmission was not the cause for the decline and disappearance of taste sensitivity described here.

In summary these results show that interruption of the CT in the middle ear makes the CT of rats insensitive to stimulation of the tongue with taste solutions within about 15 h. The interruption of the nerve may initially cause an increase in the response which then diminishes until no response can be recorded.

Discussion

The results of this study show that the response in the rat CT declines and disappears completely within 15 h after the nerve has been cut in the middle ear.

The observations described here were obtained in rats. There is no doubt that, though the general course is the same in other animals, the velocity of the decline differs. Thus in Rhesus monkeys we have recorded a response to taste stimulation in the chorda tympani nerve for more than 24 h after cutting the CT. On the other hand, the decline is faster in the

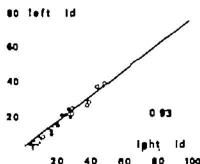


Fig. 1



Fig. 2

Fig. 1 The amplitudes of the summated response of the right and left CT nerves on the same rat ha been plotted against each other d ring stimulation with 0.3 m NaCl (□), 0 02 m citric acid (O), 0 02 quinine hydrochloride (O) and 0.3 m sucrose (■). Dat from 12 rats were used.

Fig. 2. The magnitude of the responses in the CT of 85 rats to stimulation with 0 3 m NaCl was plott against the time after the i terruption of the CT in the middle ear The diagram shows that the respo decreased with time and disappeared within 15 h. The squares (■) illustrat the response obtained af injection f a local anesthetic in the ear The horizontal line illustrates the verage magnitudes of the C of the other side The vertical bars illustrate the SD of these values.

nerve to the recording site resulted in the effects illustrated in Fig. 2, in a few animals th nerve was cut where it passes near to the tympanic membrane. The results obtained did no differ from those described. Thus the decline was not caused by local effects of alcohol the recording place

The data of Fig. 2 were based on responses to the most effective stimulus, 0.3 m NaC The slope of decline of the responses to the other stimuli did not differ and as a rule we use 4 types of stimuli.

The possible effect of general anesthesia on the decline of response

The anesthetic is another factor which may affect the decline of the response described here In an ordinary experiment the recording from the CT begins as soon as the nerve has bee cut and the dissection is made under general anesthesia It is possible that anesthesia ma slow down the processes shown in Fig. 2. Therefore in 6 rats the CT nerves on one side wa dissected and the taste response was recorded from the moment the nerve was cut unt the response disappeared. Fig. 3 shows a recording from such an animal during stimulatio with 0 1 m NaCl and 0 03 m citric acid. It shows the same feature as has been described: decline and disappearance of the response after about 12 h. The course of the decline of th individual nerve response was the same as those in Fig. 2. When the response in the C had completely disappeared, the other side was dissected and a recording was made from the CT of that side. In 3 of these 6 animals mentioned above the nerves of this side ha been interrupted with alcohol at the onset of the whole experiment In these animals n response could be recorded. In the three other animals, whose CT nerves had not bee touched earlier there was no problem of recording a normal response to taste stimulation

In summary we suggest that the decline and disappearance of the taste response after interruption of the CT was the result of an interruption of an axoplasmic flow in the gustatory fibres to the taste buds. The cessation of axoplasmic supply will probably affect the nerve endings first. As a first sign of deteriorating function a stage of hypersensitivity is a general feature.

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In summary we suggest that the decline and disappearance of the taste response after [i]nterruption of the CT was the result of an interruption of an axoplasmic flow in the gusta[tor]y fibres to the taste buds. The cessation of axoplasmic supply will probably affect the [fi]ne nerve endings first. As a first sign of deteriorating function a stage of hypersensitivity [i]s a general feature.

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Administration of adrenergic agonists and blockers. Isoprenaline (Isoprenalini sulphas, Pharm. Nord. 63) as given as an either 1 or 20 min lasting intra-arterial infusion in doses from 0.01 to 1 μg/g b.wt. Norepinephrine (noradrenalini bitartras) as given as an i.a. or i. infusion, lasting either 1 or 20 min in doses of norepinephrine) from 0.1 to 1 μg/g. As it as found that the mice often died ith signs of lung edema, hether or infusion was used, the infusions were in most cases performed after pretreatment ith 5 μg/g dihydralazine, hich causes hypotension without change of submaxillary renin release (Bing and others 1976). The changes in renin release were neither influenced by this pretreatment, nor by the route of infusion (i or) of noradrenaline, hich in most cases as i.a. Some mice received the alpha-adrenergic blocker phenoxybenzamine (Alfred Benzon) in one or repeated 1 min lasting infusions of 3 or 9 μg/g

Blood was dra from femoral artery in capillary tubes moistened with 300 mM EDTA solution. Plasma renin concentration as determined by the capture radioimmunoassay for angiotensin I (Poulsen and Jørgensen 1974) and expressed in Goldblatt Units (G.U.) 10^{-4} ml^{-1} by comparison with the Holly Hill hog renin standard, London

Results

Effect of the β-adrenergic agonist isoprenaline on release of renal and submaxillary renin

The effect of isoprenaline on renal renin release in sialoadenectomized mice is shown in Fig. 1 A and B. The maximum value of plasma renin concentration after a single infusion of 3 different doses 0.01 0.1 and 1 μg/g b.wt. is seen in Fig. 1 A, which shows a relation between dose and response. Fig. 1 B further shows that repeated infusions (indicated by

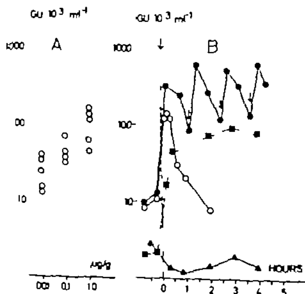


Fig. 1 *A* The dose-response relationship of the effect of isoprenaline (in μg/g b.w.) on renal renin release as shown by the maximum plasma renin concentration (in Goldblatt Units 10^{-4} ml^{-1}) in submaxillary sialoadenectomized mice. The mean preinjection renin concentration was 7.4 ± 1.1 GU 10^{-4} ml^{-1} in 34 mice. After the injection of the 3 doses the mean values ere 24.6 ± 4.8, 46.3 ± 14.8 and 101 ± 20.3 respectively, the difference between the values obtained ith the lowest and highest doses being highly significant (p 0.005). The significance between the first and the second group 0.1 p 0.05 and that between the second and the third group p 0.05. *B*. Representative examples of the time course of the changes in plasma renin produced by isoprenaline. Single infusion of 1 μg/g b.w. isoprenaline during one min (○). Repeated infusions, each of 20 min duration (●). Continuous infusion of 2.6 μg/g/h during 4 h (■). 1 μg/g isoprenaline given to nephrectomized mouse ( )

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From University Institute for Experimental Medicine, Copenhagen, Denmark

Differences in renal and submaxillary renin release after stimulation with isoprenaline and noradrenaline

By

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Abstract

JENS BING and KNUD POULSEN *Differences between the changes in renal and submaxillary renin release after stimulation with isoprenaline and noradrenaline* Acta physiol scand 1979 *105* 58–63

It is confirmed that while noradrenaline stimulates release of submaxillary as well as renal renin, isoprenaline only stimulates renal renin release The effects of these two adrenergic agonists differ in several other ways. The kidneys respond to isoprenaline with a dose dependant renin release As a contrast the submaxillary glands respond to noradrenaline by no or by non-dose-dependant release. After a single dose of the agonists the isoprenaline induced renin release is of short duration, contrasting with a prolonged renin release after injection of noradrenaline The kidneys are able to respond to repeated doses of both agonists, while the submaxillary glands most often only respond to the first dose. While the effect of noradrenaline is blocked by pretreatment with phenoxybenzamine, this blocker is without effect when given after noradrenaline The two agonists do not provoke any increase in plasma renin in mice which have been both sialoadenectomized and nephrectomized.

Key words: Adrenergic agents, renin release plasma renin

While renal renin release is stimulated by way of beta-adrenergic receptors in the kidney (see Assaykeen, 1972 and Freeman and Davis 1977), in vivo as well as in vitro experiments have shown that release of renin from the submaxillary glands is only stimulated via alpha adrenergic receptors (Menzie *et al* 1974 Michelakis *et al* 1976) The present study aims at investigating the time course of these changes in renal and submaxillary renin release and the dose response relationships for the two agonists, isoprenaline and noradrenaline.

Material and Methods

Animals. Male albino mice of the Danish State Serum Institut strain weighing about 50 g, and kept in cages containing 5 or only a single mouse. Most mice were conscious, having been placed in restraining cages after insertion of catheters in one or both femoral arteries during a short-term ether anesthesia When the agonists were given i. (in the jugular vein) the mice were under pentobarbital anesthesia (60 mg/ kg i.p.). In order to obtain basal plasma renin values the experiments were first started about 2 h after the anesthesia. Renal renin release was studied a week or more after submaxillary sialo-adenectomy and submaxillary release in mice nephrectomized 17 or more often about 2 h in advance

given by the representative curves marked O in Fig. 2 B, showing that the effect is prolonged, reaching its maximum after 1 to 2 h and staying at this level for the time of the experiment, lasting up to 6 h. In 8 expts., one of which is given in Fig. 2 B (lowest curve marked O), a second dose of 1 µg/g noradrenaline was given 3 h after the first dose, at a time when the maximum level was obtained. The second dose elicited none or only a slight further increase in plasma renin. In a ninth expt. the second dose of 1 µg/g was followed by an increase in plasma renin from about 50 to 400 GU 10^{-4} ml. When a dose of 3 µg/g phenoxybenzamine was given before noradrenaline there was the usual rise in plasma renin (N 6), but when the dose was increased to 9 µg/g the effect of noradrenaline was blocked (N 6) as shown in the curve marked ●. When doses of 3 (N = 9) or 9 µg/g phenoxybenzamine (N 6) were given 1 to 2 h after noradrenaline, there was no fall in the plasma renin concentration, the renin values staying at the high level as shown by the upper curve in Fig. 2 B.

The effect of noradrenaline on renal renin release was studied in 12 previously sialoadenectomized mice, of which 7 died of doses between 0.5 and 1 µg/g, the pre-treatment with dihydralazine in these mice being omitted because of its hypotensive effect, which causes release of renal (but not of submaxillary) renin. Of the 5 surviving mice one received a single injection of 1 µg/g noradrenaline (△ in Fig. 2 C), while the other 4 received doses from 0.25 to 0.5 µg/g, which were repeated after 2 h. As shown in Fig. 2 C the effect of noradrenaline on renal renin release differs from that obtained after infusion of isoprenaline (Fig. 1 B), the effect being prolonged, resulting in an increased level of many hours duration. And contrary to the most often lacking effect on submaxillary renin release of a second dose of noradrenaline, the second dose resulted in all cases in a marked further increased renal renin release, as shown by representative example marked △ in Fig. 2 C, in which the second injection is indicated by an arrow marked N. The renin concentration rose from a mean preinjection value of 7.8 ± 0.9 to 28.6 ± 5.4 GU 10^{-4} ml ($p < 0.01$) after the first injection (N 5) and from 18.8 ± 2.5 to 95.3 ± 30 (p 0.025) after the second injection (N 4).

II. *Effect of isoprenaline and noradrenaline on the plasma renin concentration in mice after removal of submaxillary glands as well as kidneys*

When the agonists in doses of 1 µg/g were given to mice which after previous sialoadenectomy had been nephrectomized about 2 h in advance, it was found, that neither isoprenaline (N 8) nor noradrenaline (N 8) provoked any increase in plasma renin concentration.

Discussion

The results confirm the findings of Menzie *et al.* (1974) and Michelakis *et al.* (1976), showing that the beta-adrenergic agonist isoprenaline causes a release of renal, but not of submaxillary renin, that the alpha-adrenergic agonist noradrenaline causes renin release from both kidneys and submaxillary glands, and that the latter effect of noradrenaline is blocked by phenoxybenzamine. Both agonists are shown to be unable to provoke an increase in plasma renin concentration in mice, which have been sialoadenectomized as well as nephrectomized.

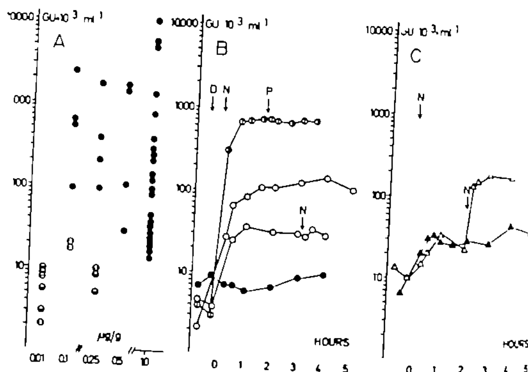


Fig. 2. *A* The effect of different doses of noradrenaline (in μg/g) on submaxillary renin release as shown by the maximum plasma renin concentration (in GU 10^{-4} ml^{-1}) in 35 nephrectomized mice. ⊖ mark the cases in which the plasma renin concentration was uninfluenced by the injection and ● the cases where it was higher after injection of noradrenaline than before the injection. There is no dose-response relationship (p > 0.05). *B* Representative examples of the time course of the changes in plasma renin concentration after single doses of 1 μg/g noradrenaline (arrow marked N) infused during 1 min after pre-treatment with dihydralazine (D) in nephrectomized mice (O). In one case (indicated by a second arrow marked N) the infusion was repeated. In the experiment given by (●) 9 μg/g of phenoxybenzamine was given before noradrenaline. In the experiment marked by ⓘ the same dose was given after noradrenaline at the time indicated by the arrow P. *C* The time course of changes in plasma renin concentration due to the renal renin release after a single infusion of 1 μg/g noradrenaline in a sialo-adenectomized mouse (▲), and the effect of repeated infusions of doses of 0.25 μg/g in a sialo-adenectomized mouse (△).

arrows) resulted in repeated increases in plasma renin (N = 7), and that continued infusion of 2.6 μg/g/h during several hours (■) resulted in a rather constant high level (N = 11). The figure finally shows that 1 μg/g isoprenaline does not cause any release of submaxillary renin when infused in nephrectomized mice (N = 3, marked ▲).

II *Effect of the predominantly α-adrenergic agonist noradrenaline on release of submaxillary and renal renin*

The effect of noradrenaline on the release of submaxillary renin was studied in nephrectomized mice, the doses being between 0.01 and 1 μg/g b.wt. given *i.a.* during 1 min after pre-treatment with dihydralazine. The results are seen in Fig. 2 A and B. The maximum value for plasma renin concentration after a single injection of different doses, 0.01, 0.1, 0.25, 0.5 and 1 μg/g b.wt. is given in Fig. 2 A. It is seen that while a dose of 0.01 μg/g was without effect on plasma renin, doses of 0.1 to 1 μg/g in most cases resulted in increases with marked differences between the individual maximum values, but without any significant difference between those obtained with the four doses. The time course of the changes in plasma renin

given by the representative curves marked ○ in Fig. 2 B, showing that the effect is prolonged, reaching its maximum after 1 to 2 h and staying at this level for the time of the experiment, lasting up to 6 h. In 8 expts., one of which is given in Fig. 2 B (lowest curve marked ○), a second dose of 1 µg/g noradrenaline was given 3 h after the first dose, at a time when the maximum level was obtained. The second dose elicited none or only a slight further increase in plasma renin. In a ninth expt. the second dose of 1 µg/g was followed by an increase in plasma renin from about 50 to 400 GU 10^{-6} ml^{-1} When a dose of 3 µg/g phenoxybenzamine was given before noradrenaline there was the usual rise in plasma renin (N 6), but when the dose was increased to 9 µg/g the effect of noradrenaline was blocked (N 6) as shown in the curve marked ● When doses of 3 (N = 9) or 9 µg/g phenoxybenzamine (N 6) were given 1 to 2 h after noradrenaline, there was no fall in the plasma renin concentration, the renin values staying at the high level as shown by the upper curve in Fig. 2 B

The effect of noradrenaline on renal renin release was studied in 12 previously sialoadenectomized mice, of which 7 died of doses between 0.5 and 1 µg/g, the pre-treatment with dihydralazine in these mice being omitted because of its hypotensive effect, which causes release of renal (but not of submaxillary) renin. Of the 5 surviving mice one received a single injection of 1 µg/g noradrenaline (△ in Fig. 2 C), while the other 4 received doses from 0.25 to 0.5 µg/g, which were repeated after 2 h. As shown in Fig. 2 C the effect of noradrenaline on renal renin release differs from that obtained after infusion of isoprenaline (Fig. 1 B), the effect being prolonged, resulting in an increased level of many hours duration. And contrary to the most often lacking effect on submaxillary renin release of a second dose of noradrenaline, the second dose resulted in all cases in a marked further increased renal renin release, as shown by a representative example marked △ in Fig. 2 C, in which the second injection is indicated by an arrow marked N. The renin concentration rose from a mean preinjection value of 7.8 ± 0.9 to 28.6 ± 5.4 GU 10^{-6} ml^{-1} ($p < 0.01$) after the first injection (N 5) and from 18.8 ± 2.5 to 95.3 ± 30 ($p = 0.025$) after the second injection (N 4).

(III) *Effect of isoprenaline and noradrenaline on the plasma renin concentration in mice after removal of submaxillary glands as well as kidneys*

When the agonists in doses of 1 µg/g were given to mice which after previous sialoadenectomy had been nephrectomized about 2 h in advance, it was found, that neither isoprenaline (N 8) nor noradrenaline (N 8) provoked any increase in plasma renin concentration.

Discussion

The results confirm the findings of Menzie *et al* (1974) and Michelakis *et al.* (1976), showing that the beta-adrenergic agonist isoprenaline causes a release of renal, but not of submaxillary renin, that the alpha-adrenergic agonist noradrenaline causes renin release from both kidney and submaxillary glands, and that the latter effect of noradrenaline is blocked by phenoxybenzamine. Both agonists are shown to be unable to provoke an increase in plasma renin concentration in mice, which have been sialoadenectomized as well as nephrectomized.

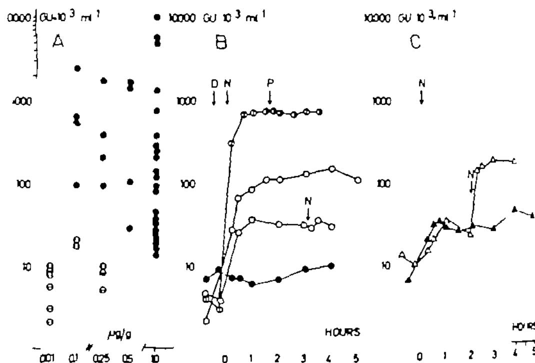


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This finding shows the correctness of the assumption of Menzie *et al.* (1974), that the creases in renin concentration in sialoadenectomized and in nephrectomized mice effe by the two agonists were due to release of renal and submaxillary renin respectively

It is further shown that the effects of these two adrenergic agonists differ in several wa While there is a rather good dose-response-relationship of the effect on plasma renin co centration after injection of isoprenaline on sialoadenectomized mice with intact kidne (Fig. 1 A), no such relationship is seen in noradrenaline treated nephrectomized mice w intact submaxillary glands, as shown in Fig. 2 A, which further shows the marked d ferences in the individual responses to noradrenaline.

Further differences are seen in the time course of the changes in plasma renin concentr tion that produced by isoprenaline (due to renal renin release) being short (Fig. 1 B), wh noradrenaline causes a change in submaxillary renin release, resulting in a prolonged i crease in plasma renin, lasting more than 6 h This continued high level can be due to co tinued release of submaxillary renin. The finding that doses of phenoxybenzamine, wh block the effect of noradrenaline, when given before this agonist, are without effect, wh given after the noradrenaline (Fig 2 B), could be due to a similar mechanism, as that caus the similarly continued high level found after gentle manipulation and subsequent remo of the submaxillary glands in nephrectomized mice In these it was found that the contin high level of plasma renin concentration was due to transfer of renin with submaxilla lymph or interstitial fluid (perhaps with renin containing tubular granules) to surroundi tissues with a subsequent slower release from these tissues to the blood (Bing *et al.* 197 The lack of effect of phenoxybenzamine on the high renin level when given after no adrenaline, can, however also be due either to inability of the phenoxybenzamine to d place the agonist from the receptors, or more likely to an inability of the blocker to influen the secretion after the latter has been stimulated. The prolonged increase in plasma re which was found after a single injection of noradrenaline both in the nephrectomiz (Fig. 2 B) and in the sialoadenectomized mice (Fig. 2 C) can however also be due eithe release of inactive renin, which is activated with time, or to a protracted release due to t severe circulatory stress caused by treatment with noradrenaline.

Renal and submaxillary renin differ in their ability to respond to repeated doses of t adrenergic agonists. The renal renin was released each time isoprenaline (Fig. 1 B) or no adrenaline (Fig. 2 C) was given while submaxillary renin in all but one case was only r leased following the first dose (Fig. 2 B) This difference may be related to the finding the marked individual, non-dose dependent responses of submaxillary renin release (Fi 2 A), which contrasts to the dose-dependency of the response of renal renin release to is prenaline (Fig. 1 A).

This study was supported by grants from the D nish Heart Foundation F L. Smidth & Co. Foundati King Christian X. Foundation nd the Foundation of the Insurance Compa ies of 1952. The Selektro Analyser Model 45-23 used for the radioimmunoassay is a gift from the Danish St te Medical Resea Council The authors are grateful to Alfred Benzon, Copenhagen, for the generous gift of phenoxy ben mine Dr Ole Fredriksen kindly performed the statistical calculations.

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In mice aggressive behaviour provokes vast increase in plasma renin concentration, causing only slight, if any, increase in blood pressure

By

JENS BING and KNUD POULSEN

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Abstract

BING J and K. POULSEN *In mice aggressive behaviour provokes vast increase in plasma renin concentration causing only slight if any increase in blood pressure* Acta physiol. scand 1979 *105* 64–72.

In mice aggressive behaviour causes a vast release of renin, which can result in about 600-fold increase plasma renin concentration, reaching 6 Goldblatt Units, corresponding to 15 μg renin per ml. This increase is mainly due to release of submaxillary renin, but there is also a significantly increased renal renin release. The degree of renin release is influenced by the duration of the aggression and by previous contact with other mice. Contrasting with the vast increase in plasma renin the blood pressure is normal or only moderately increased. This disproportion is not due to the depletion of renin substrate caused by the increased renin, as shown by the increased calculated renin activity as well as by decrease in blood pressure elicited by blockade of the renin system. Nor is the disproportion due to change in the sensitivity of the vessels to angiotensin II, the cause of this lack of tachyphylaxis being unknown. By way of exclusion the lack of pronounced increase in blood pressure can be explained by homeostatic function of the cardiovascular reflexes, which may also account for the fact that the pressor response after injection of pure submaxillary renin is only short, contrasting with a prolonged marked increase in plasma renin concentration.

Key words. Aggressive behaviour renin release blood pressure

Renal renin release is known to be influenced by several factors (see Freeman and Davis 1977), and in mice renin is released from the submaxillary glands by stimulation with alpha adrenergic agonists (Menzie *et al* 1974) It is, therefore, necessary to control the circumstances under which blood is collected for determination of basal plasma renin concentration and activity as rigorously as possible (see Pettinger *et al* 1975) In mice we have previously aimed at having "standard conditions" by using conscious mice kept in restraint cages after insertion of a catheter in a femoral artery during a shortterm ether anesthesia. This pretreatment produced a primary increase in plasma renin concentration, which generally returned to "basal values" after about 2 to 3 h (Bing and Poulsen 1975). Some of these mice, however had markedly higher renin concentration, the cause of which was unknown. In continued experiments the occurrence of such exceptions was confirmed

as being the case in normal, nephrectomized as well as in sialoadenectomized mice. An idea of the cause of these exceptional cases with high values was obtained when plasma renin concentrations about 20 to 200-fold the usual basal values were found in 3 mice, which had been kept in social contact and which had been vigorously fighting before the start of the experiment. Based on this observation the aim of the present investigation was to study the effect of aggressive behaviour on the release of renal and submaxillary renin. The aggression was caused by social contact between normal, sialoadenectomized or nephrectomized previously individually housed mice. For comparison the plasma renin concentration was determined in isolated mice, which had not been confronted with other mice. Besides plasma renin also renin substrate concentration, renin activity and blood pressure were determined in several of the mice. In some of these the response of the cardiovascular system to angiotensin II and noradrenaline as well as the effect of blockade of the renin system on the blood pressure were also studied.

Material and Methods

Animals. Male albino mice of the Danish State Serum Institute strain, weighing 50–70 g, individually housed in cages (internal size: 27 × 15 × 9.5 cm) from 2 to 30 days were used for determination of plasma renin concentration, the differences in time of isolation being without influence on the values. Most mice were conscious during sampling of arterial blood as described in the Introduction, but in some mice blood from the tail vein was sampled during 1–2 min lasting light ether anesthesia, the type of sampling had no significant influence on the results.

Aggressive behaviour was obtained by grouping 3 mice, which had previously been housed individually for at least 1 month. A pre-fighting between the animals had previously been provoked (after 14 days isolation), as this has been found to intensify the aggressive behaviour (Kreuk and Jenkin 1969). The grouping took place in a small cage (13 × 13 × 9.5 cm), in which the mice were kept for varying times, as described in the following, the fighting behaviour being recorded in all experiments (see Valzelli 1969 and Lagerspetz 1969). In some expts. groups of 3 mice were placed in similar small cages for 10 min three times each for at least 1 month before their aggressive behaviour and its influence on renin release and blood pressure were tested. Some mice were normal, others had been submaxillary sialoadenectomized at least a week and most often several weeks before, and a few mice were nephrectomized during a short-term ether anesthesia 2 h before the grouping.

Determination of plasma renin concentration and renin substrate and calculation of the plasma renin activity were performed as described in a recent paper (Bing and Poulsen 1977), using the capture radioimmunoassay of Poulsen and Jørgensen (1974). Conversion of Goldblatt Units × 10^{-4} (expressed by comparison with the Holly Hill hog renin standard, London) to ng renin was performed by multiplication with 44, based on the finding that the specific enzymatic activity of submaxillary and renal renin is 0.41 × 10^{-4} Goldblatt Unit × 1 ng^{-1} (Malling and Poulsen 1977 b). *Blood pressure* was recorded with a Tybjærg Hansen transducer and a Servogor 511 recorder. The pure submaxillary renin used for injection, was that purified by Malling and Poulsen (1977). *Blockade of the angiotensin system* was performed by an infusion of 3 (to 6) mg kg⁻¹h⁻¹ of the competitive angiotensin II inhibitor Saralasin (Norwich Pharm. Comp.). *Response of the cardiovascular system to angiotensin II* was examined by determination of the blood pressure responses to synthetic Asp¹-Ile⁵-ang. II (Schwartz Bio Res.) which were compared with those elicited by noradrenaline.

Results

1) *Plasma renin concentration in mice which are individually housed in order to avoid aggressive behaviour.* When given in Goldblatt Units × 10^{-4} × ml^{-1} the mean plasma renin was 9.8 ± 5.1 in 17 normal, 7.9 ± 3.2 in 25 at least 1 week earlier sialoadenectomized, 3.5 ± 1.7 in 16 mice 2 h after nephrectomy and about the same: 3.1 ± 1.8 in 10 mice nephrectomized

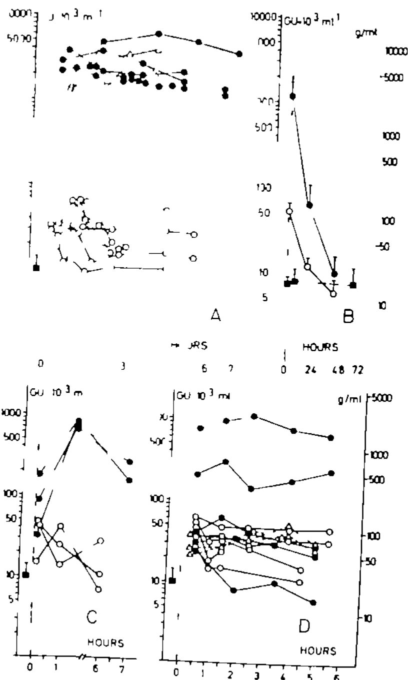


Fig. 1 *A* The time course f the plasma renin concentrations fter prolonged (30 mi) ggressive behaviour in normal (●), nephrectomized (△) and submaxillary sialoadenectomized (○) mice during the first 3 to 7 h. These increases are all significant (P 0 001) *B*. Mea values from similarly treated mice (6 normal and 6 sialoadenectomized) during 48 t 72 h. The f g. ncludes the mean values from 3 normal mice not provoked to aggression (■). The lines connecting the values re drawn without consideration the primary plateau seen in Fig 1 A *C*. Plasma re in concentrations observed after only 3 min ggressive behaviour *D* Responses f und in mice which before prolonged aggressive behaviour had been pre treated for about a month with repeated confrontations.

The symbols are the same in all f gures. The left ordinated indicat the plasma renin concentrations G U 10⁻³ ml⁻ and the right rdinates the same values fter conversion to g/ml given on a logarit mic scale. The abscissae show the time in hours after the start of the conf ontation, which is marked by vertical dotted line.

7 h earlier and sialo-adenectomized at least 1 week earlier. Two values which differed markedly from the mean concentrations were excluded, 59 GU 10^{-4} ml^{-1} found in plasma of a normal mouse and 39 found in that of a sialoadenectomized mouse.

2. *Effect of prolonged pronounced aggressive behaviour on plasma renin concentration and subsequent changes in plasma renin substrate concentration and renin activity.* During grouping for 30 min of previously isolated mice the type of aggressive behaviour was the same in normal and sialoadenectomized mice. The fighting behaviour exploded immediately with powerful attacks, which were repeated up to 5 times a minute or consisted of up to 20 s lasting fight, most often including all 3 mice. In the last 10 min the attacks were less fierce and other types of the aggressive pattern, such as nosing, tail rattling and squeaking were observed. In the nephrectomized mice the aggressive behaviour started with these types, but after 2 min the first real attack was observed. The aggression was, however, less pronounced in these mice.

In 10 normal mice the aggressive behaviour caused a vast increase in plasma renin concentration, which was about 100 to 600-fold the normal and stayed at these high levels for 6 h (● Fig. 1 A). As shown by the mean values (● in Fig. 1 B) from 6 other normal mice studied for 48 h after the aggression, normal values were first reached after about 42 h. In 3 two-hours previously nephrectomized mice (△ Fig. 1 A) the renin concentration was only questionably lower than after aggression in the normal mice. 8 sialoadenectomized mice had also increased renin concentration, which, however, was only about 2 to 10 times the normal values. In several of these mice the renin concentration declined during the 3 to 6 h lasting expts. (○ Fig. 1 A). As shown by the mean values (○ in Fig. 1 B) from 6 other sialoadenectomized mice normal values were reached after about 24 h. Fig. 1 B further shows the mean of values from 3 normal mice, which had not been made aggressive (marked ■). These values agree with the above mentioned mean from 17 other normal mice.

The effect of the vast aggression-provoked increase in plasma renin concentration on plasma renin substrate and renin activity was studied in 8 of the normal mice at the end of the experiments shown in Fig. 1 A. While the mean renin substrate concentration in 8 normal controls was 363 ng/ml AI (range 202–448), there was a marked depletion of the substrate in the aggressive mice, in which the mean value was 58 (range 24–90). The corresponding calculated mean renin activity was 3 ng AI/ml/h (range 1.2–4.7) in the 8 controls and 17? (range 45–295) in the 8 experimental mice, in which it was determined about 5 h after prolonged aggression. An other sign of presence of angiotensin in the blood was obtained by blockade of the renin system with the competitive angiotensin II inhibitor Saralasin (Fig. 2 D). In 5 normal mice, which after prolonged aggressive behaviour had a vast increase in plasma renin concentration (mean 1 350 GU 10^{-4} ml^{-1}), Saralasin infusion resulted in a mean decrease of the blood pressure of 23 mmHg (range 11 to 32 mm).

3. *Effect of short pronounced aggressive behaviour on plasma renin concentration* was studied in 3 normal and in 3 sialoadenectomized mice. In these studies pretreatment and grouping was performed as described above, but the animals were separated after 3 min fighting. In the 3 normal mice the plasma renin concentration was markedly increased immediately after the aggression (Fig. 1 C) and still much higher 90 min later, at which time they however did not reach the values found after prolonged aggression (Fig. 1 A). Contrary to the

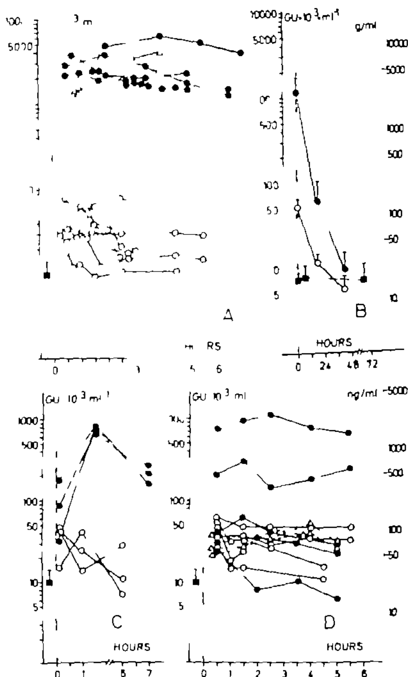


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Effect of prolonged pronounced aggressive behaviour on plasma renin concentration and bsequent changes in plasma renin substrate concentration and renin activity. During grouping 30 min of previously isolated mice the type of aggressive behaviour was the same in ormal and sialoadenectomized mice. The fighting behaviour exploded immediately with owerful attacks, which were repeated up to 5 times minute or consisted of up to 20 s sting fight, most often including all 3 mice. In the last 10 min the attacks were less fierce nd other types of the aggressive pattern, such as nosing, tail rattling and squeaking were bserved. In the nephrectomized mice the aggressive behaviour started with these types, ut after 2 min the first real attack was observed. The aggression was, however less pronounced in these mice.

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The effect of the vast aggression-provoked increase in plasma renin concentration on lasma renin substrate and renin activity was studied in 8 of the normal mice at the end of he experiments shown in Fig. 1 A. While the mean renin substrate concentration in 8 ormal controls was 363 ng ml AI (range 202–448), there was a marked depletion of he substrate in the aggressive mice, in which the mean value was 58 (range 24–90). The orresponding calculated mean renin activity was 3 ng AI/ml/h (range 1.–4.7) in the 8 ontrols and 172 (range 45–295) in the 8 experimental mice, in which it was determined bout 5 h after prolonged aggression. An other sign of presence of angiotensin in the blood as obta ned by blockade of the renin system with the competitive angiotensin II inhibitor Saralasin (Fig. D). In 5 normal mice, which after prolonged aggressive behaviour had st increase in plasma renin concentration (mean 1 330 GU 10^{-4} ml^{-1}), Saralasin in-fusion resulted in a mean decrease of the blood pressure of 23 mmHg (range 11 to 32 mm).

3 *Effect of short pronounced aggressive behaviour on plasma renin concentration* was studied in 3 normal and in 3 sialoadenectomized mice. In these studies pretreatment and grouping was performed as described above, but the animals were separated after 3 min fighting. In the 3 normal mice the plasma renin concentration was markedly increased immediately after the aggression (Fig. 1 C) and still much higher 90 min later at which time they how ever did not reach the values found after prolonged aggression (Fig. 1 A). Contrary to the

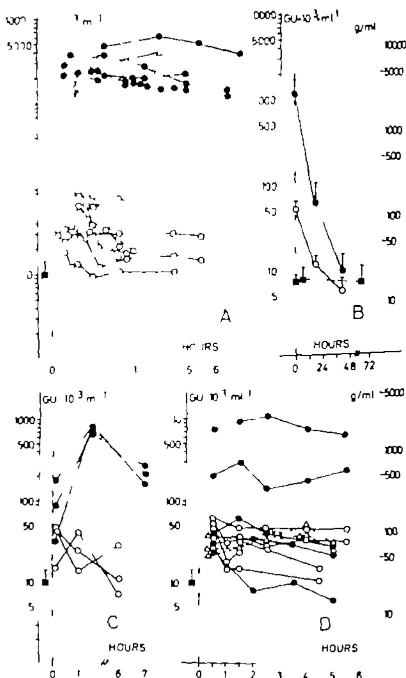
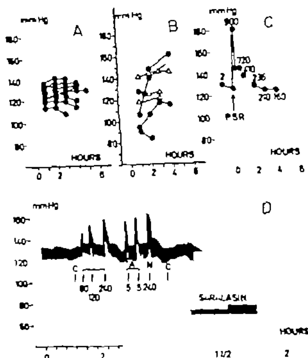


Fig. 1. *A.* The time course f the plasma renin concentrations after prolonged (30 min) aggressi behaviour in normal (●), nephrectomized (Δ) and submaxillary sialoadenectomized (○) mice during the first 3 to 7 h. These increases are all significant (P 0.001). *B.* Mea values from s milarly treated mice (6 normal and 6 sialoadenectomized) during 48 to 72 h. The f g ncludes the mean values from 3 normal mice not provoked to aggression (■). The l nes connecting the values are drawn without consideration of the primary plateau seen in F g. 1 A. *C.* Plasma reni concentrations bserved after only 3 min aggressive behaviour. *D.* Responses found i mice which before prolonged ggressi beha iou had been pre treated for about month with repeated confrontations.

The symbols are the same all f gures. Th left ordinated indicat the plasma re in concentrations in G U × 10^{-3} ml^{-1} and the right ordinates the same values fter conversion to g/ml given on a logarithmic scale. The abscissa show the time in hours fter the start of the confrontation, which is marked by vertical dotted line.



2. *A*. Blood pressures in normal, non-pre-treated mice and *B*. blood pressures after prolonged aggressive behaviour in normal (●) and nephrectomized (Δ) mice with vast increases in plasma renin concentration (as shown in Fig. 1 A). *C*. The effect on the blood pressure of sialoadenectomized and nephrectomized mouse of injection of 5.6 G.U pure submaxillary renin (P.S.R.); the corresponding plasma renin concentrations are given in G.U. 10^{-4} ml^{-1} by figures at the curve. *D*. Blood pressure tracing in mouse with vast increase in plasma renin after prolonged aggressive behaviour. Pressor responses shown for angiotensin II (A, doses given in ng) and noradrenaline (N, doses in ng). C indicates control injection of physiological saline. The latter part of the tracing shows the depressor effect of Saralasin infusion (initially 3, later 6 μg kg^{-1} h^{-1}).

mice the mean blood pressure was 125 mmHg (range 108–145) with only small (max. 10 mm) variations with time in the individual mouse.

For comparison with the relation between plasma renin concentration and blood pressure in aggressive mice the same relation was investigated in 5 sialoadenectomized and nephrectomized mice, in which the two parameters were measured before and after intraarterial injection of pure submaxillary renin (P.S.R.). A representative example of the nearly identical results of these experiments is given in Fig. 2 C. Although the renin concentration, as shown by the figures given at the curve, rose from 2 to 900 GU 10^{-4} ml^{-1} and was still about 80-fold increased after 4 h, a marked increase in blood pressure was only present for about 15 min. It was below the upper limit of the normal (140 mmHg) after 1 h, reaching the pre-injection level about 2 h after the injection, at a time when the renin concentration was still well over 100-fold increased.

6. Cardiovascular reactivity to the pressor action of angiotensin II in mice with vast increase in plasma renin concentration and activity caused by aggressive behaviour. 5 normal mice, in

constant high values found after prolonged aggression, the values had fallen marked[ly at] the end of the 7 h lasting experiment. In the 3 sialoadenectomized mice the short aggres[sion] resulted in an increase in plasma renin, which was similar to the lowest values found [af]t. prolonged aggression.

4 *The influence of repeated confrontations on the type of aggressive behaviour and on plas[ma] renin concentration* was studied in 9 normal mice, one of which died when it was anesthetiz[ed] after the last confrontation and in 6 sialoadenectomized mice.

After 3 weeks isolation groups of 3 of these mice were brought in contact for 10 [m]. three times a week for 4 to 6 weeks. At the start of this period the type of aggressive b[e]haviour was the same as previously described, but in all 9 + 6 mice the aggressiveness [de]clined with time This was especially the case in the normal mice, which for the last half [of] the month started with nosing and tail rattling, powerful attacks first taking place after 3[–] 5 min contact. Even then the attacks were less frequent and were of shorter duration th[an] in the first confrontations. After this pretreatment the 3 mice of each group were confront[ed] for about 20 min during which the level of aggression was decreased as in their last fighting 3 of the normal mice had been nephrectomized 2 h before this confrontation. In the[se] there was no signs of aggression, and even addition of 2 normal mice elicited only a fe[w] attacks.

The effect of this decreased aggression on plasma renin concentration (Fig. 1 D) w[as] about the same in the sialoadenectomized (○) nephrectomized (△) and in 3 of the 5 norm[al] (●) mice, the values being between 25 and 60 GU 10^{-4} ml In two of the normal m[ice] higher values were found but they did not reach the level observed following aggression [in] normal mice, not subjected to the above mentioned pretreatment (● Fig. 1 A).

5 *Effect of the vast increase in plasma renin concentration on the blood pressure* The bloo[d] pressure was followed for up to 4 h in 5 of the normal and in the 3 nephrectomized m[ice] in all of which the renin concentration after the aggression rose to and stayed above 10[0] GU 10^{-4} ml as shown in Fig. 1 A (● and △). While the blood pressure of 10 contr[ol] mice with normal plasma renin was between 110 and 140 mmHg with only small chang[es] (maximum 10 mm) with time (Fig. 2 A), the blood pressure of the 8 mice with vast increa[se] in plasma renin was more variable, as seen in Fig. 2 B (in which normal mice are marked ● nephrectomized △). This was both due to more varying blood pressure values with time an[d] to more marked individual differences. The blood pressure did, however only exceed th[e] maximum level of the normal (140 mmHg) in three mice, in which the values reached 14[?] 154 and 160 mm respectively

The time course of the blood pressure of sialoadenectomized mice was studied in the [?] mice in which aggressive behaviour had resulted in renin concentrations from about [?] to 100 GU 10^{-4} ml (○ Fig. 1 A) The mean blood pressure of these mice was identic[al] with that of 7 sialoadenectomized mice, which had not been subjected to aggression, th[e] mean values in both being 123 mmHg (range 110–140) with only small (max. 12 mm), [if] any variation with time in the individual mouse.

The time course of the blood pressure was finally followed for 4 h in 3 of the normal an[d] the 6 sialoadenectomized mice after the aggression which followed the repeated fightin[g] 3 times a week during 4 to 6 weeks, the renin values of which are shown in Fig. 1 D In the[se]

3 *Effect of the vast increase in plasma renin concentration on blood pressure.* The blood pressure of normal isolated mice was between about 110 and 140 mmHg (Fig. 2 A), not differing from that previously found in mice kept in social contact (Bing and Poulsen 1975). In pronounced contrast to the vast increases in plasma renin concentration found after aggression the blood pressures were only somewhat above the maximum level of the normal in a few mice. However the blood pressure was more variable with time and between individuals (Fig. 2 B). The marked disproportion between plasma renin and blood pressure is similar to that previously found after manipulation of the submaxillary glands (Bing and Poulsen 1977), and its cause can be the same in these two groups of differently prepared mice. Also in the present study the only small, if any increase in blood pressure could not be attributed to a demonstrated significant depletion of renin substrate, as this was actually associated with a marked increase in calculated renin activity. A direct demonstration of formation and function of angiotensin II in the post-aggressive period was obtained by the finding of a significant decrease in blood pressure after blockade of the renin-system with the competitive angiotensin II inhibitor Saralasin (Fig. 2 D). The disproportion between plasma renin and blood pressure was further found not to be due to reduced responsiveness (tachyphylaxis) of the resistance vessels to angiotensin II, as the animals showed a normal response to angiotensin II (Fig. 2 D), a surprising finding in animals with such vast increases in plasma renin concentration. Such lack of tachyphylaxis was also found in about half of the mice with similarly high renin values after manipulation of the submaxillary glands. After aggression as well as after manipulation, release of submaxillary kallikrein or another depressor substance which could counteract the effect of the increased release of renin, cannot be excluded. That disproportion between renin and blood pressure occurs even in experiments, in which release of such a hypothetical submaxillary depressor substance can be excluded, is shown by the experiments on sialoadenectomized as well as nephrectomized mice, in which injection of pure submaxillary renin resulted in prolonged marked increase in plasma renin with an only short-lasting increase in blood pressure (Fig. 2 C). As the disproportion between plasma renin and blood pressure could not be explained in any of the previously discussed ways it must for the present be believed that the lack of increase in blood pressure is due to homeostatic function of slow compensatory cardiovascular reflexes (see Day et al. 1965). However such compensation does not make itself apparent after application of a Goldblatt clamp whence an increase in plasma renin is accompanied by a marked increase in blood pressure.

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Discussion

1 *The plasma renin concentrations of individually housed mice* were found to be the same as those previously found in mice kept in social contact (Bing and Poulsen 1975) the concentrations in the sialoadenectomized being insignificantly lower than in the normal while *markedly lower values were found in nephrectomized mice and after removal of kidneys* as well as submaxillary glands. Abnormally high values were more seldom found in isolated mice than in mice kept in social contact the few high values found showing that other causes than aggression can result in increased renin release.

After *conversion of the concentrations of enzymatically active renin from Goldblatt units to ng renin per ml* the mean values in normal and sialoadenectomized mice were 24 to 1[illegible] respectively which agrees well with the values of 23 and 21 ng found by Menzie *et al.* (1974) by radioimmunoassay for the direct measurement of renin. 17 h after nephrectomy of 11 previously sialoadenectomized mice the calculated mean concentration was 7.5 ng/ml which is lower than the mean value of 11 ng/ml found by Menzie *et al* in 10 mice 24 h after removal of submaxillary glands as well as kidneys.

2. *Effect of aggressive behaviour on renal and submaxillary renin release* The study shows that prolonged aggressive behaviour in mice results in vast increase in plasma renin concentration *reaching values between 1 500 and 6 000 GU 10^{-4} ml (corresponding to* 3 7 to 15 μg/ml) and thus well over 150 to 600-fold the normal concentration (● in Fig. 1 A) These maximum values are mainly due to release of submaxillary renin, as shown by the only little lower concentrations found in nephrectomized mice (△ in Fig. 1 A), by the markedly lower concentrations found in sialoadenectomized mice (○ in Fig. 1 A) and by our previous finding of a similar vast increase after gentle manipulation of the submaxillary glands (Bing and Poulsen 1977) That the aggression also causes release of renal renin is made probable in the experiments on sialoadenectomized mice (○ Fig. 1 A) the plasma renin here being about 2 to 10-fold increased. In these mice participation of an extra-renal release can however not be excluded

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From the Institute of Medical Physiology B, University of Copenhagen, Denmark

Capillary permeability measured by bolus injection, residue and venous detection

By

PER SEJRSEN

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Abstract

SEJRSEN, P. *Capillary permeability measured by bolus injection, residue and venous detection.* Acta physiol. scand. 1979 *105* 73–92.

Kinetic analysis of residue and outflow curves of γ-emitting indicators such as chromium-51 EDTA and iodo-131-thalamate from skeletal muscle gives the possibility to determine the extraction fraction and the plasma flow and from these two values the capillary diffusion capacity can be calculated (Sejrsen 1970, preliminary report). This is possible both for the transport from blood to tissue and from tissue to blood. This alternative method has been compared in the autoperfused cat gastrocnemius preparation with the indicator diffusion method based on venous registration of a diffusible test indicator and an intravascular reference indicator (Chinard *et al.* 1955, Crone 1963). The results of the five independent measurements show good agreement. Calculation of the permeability P_d based on a capillary surface area of 7 000 cm²/100 g of tissue gives values of 1.05 · 10⁻⁵, 1.10 · 10⁻⁵ and 1.16 · 10⁻⁵ cm/s, which is in agreement with results obtained by other investigators. The permeability was equal in both directions, and thus the capillary membrane seems to function as a symmetrical membrane. Using an area of 5 000 cm²/100 g which presumably is more relevant in the plasma flow range used gives P values around 1.5 · 10⁻⁵ cm/s. The effective pore area is calculated to constitute 1/50 000 of the capillary surface area. Calculation of volumes of distribution in the muscle tissue gave intravascular plasma volumes of 1.8 to 2.0 ml/100 g, an extravascular volume of 12.4 and 15.2 ml/100 g and a final monoexponential component constituting a compartment of 5.4 and 7.0 ml/100 g from residue and venous curves, respectively. The last mentioned compartment constitutes nearly 50 per cent of the extravascular space, and it is suggested, that it is located inside the sarcoplasmic reticulum, which anatomically constitutes about 50 per cent of the interstitial space. The total area of contact between the longitudinal and the transversal tubules in this subsystem, which is the membrane of the lateral saccus, is estimated to about 6 times the capillary surface area at a plasma flow of 15 ml/100 g min, which gives a permeability about 60 times lower for this membrane compared to the capillary membrane.

Measurement of capillary permeability has been attempted by various techniques (Pappenheimer, Renkin and Borrero 1951, Chinard, Vosburg and Enns 1955, Crone 1963). Some of these techniques have been criticized with respect to their theoretical basis (Crone 1970). The present article presents an alternative technique which is based on intra-arterial bolus injection of a γ-emitting indicator and residue or venous detection (Sejrsen 1970, preliminary report). The injected indicator serves as its own intravascular reference and permeability data are obtained by kinetic analysis of the recorded response function. In the present article a more detailed treatment of the theoretical basis of the method has been performed.

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5 Inefficiency counting, i.e. equal counting efficiency for individual fractions of the indicator dose throughout the passage through the vessel and the tissue area seen by the detector but there is no demand of equal counting efficiency from one tissue region to other

6 Recirculation is eliminated, negligible or known.

7 The height of the registered curve is a relative measure of the total amount of indicator injected, i.e. all indicator molecules must be present in the field seen by the detector at a certain time

8 The early part of the curve for the extracted fraction must be deducible from the later part of the curve, i.e. the washout of the extracted fraction can be simulated by some mathematical function and extrapolated retrogradly to peak time yielding a relative measure of the extracted amount of indicator as the numerical value of the retropolated curve at peak time This time is the time where the total amount of indicator is present in the counting field and therefore also the time where total amounts of the two fractions is present. The retropolation can be performed from a time where a vascular transit after a bolus injection of a vascular indicator has come to an end for all practical purposes.

If these assumptions are fulfilled the extracted fraction, E(res), can be calculated from the residue detection curve as the height of the curve representing the transit of the extracted fraction, H(ev), obtained by retropolation to peak time, divided by the height of the registered curve, H(total)

$$E(res) \quad \frac{H(total)}{H(ev)} \tag{1}$$

The curve of the transmitted fraction, T obtained by numerical subtraction of the extrapolated curve from the detected curve allows calculation of the mean transit time of this fraction and if the vascular volume and the hematocrit are known plasma and blood flow can be computed. Experimentally it is possible to validate the performed curve resolution by comparison of the results to plasma flow calculated from directly measured venous outflow and the curve configuration to that of an intravascular indicator

Extraction from tissue to blood

In this connection the extracted fraction, E, means the fraction exchanged from tissue to blood related to that obtainable at equilibrium over the capillary membrane.

When followed to infinity the residue curve contains information of indicator permeation in the tissue to blood direction. To obtain this information the nine assumptions mentioned above must be fulfilled together with the condition

9 That the total area under the curve from time zero to infinity can be estimated.

The mean transit time, $\bar{t}$, for an indicator through a system is the mean value of transit times for the indicator particles.

$$\bar{t} = \frac{t_1 \; t_2 \; t_3 \; \ldots \; t_n}{n} \tag{2}$$

where $t_1, t_2, t_3, \ldots, t_n$ denote the transit times of each particle, and n is the total number of particles.

Eq.

To evaluate this method experimentally it was used simultaneously with the indicator dif sion method which employs a venous sampling technique and simultaneous bolus injec of a permeating test substance and an intravascular reference substance (Chinard *et al.* 19 Crone 1963)

The method presented uses uptake as well as washout principles and measures the tr capillary extraction fraction in both directions over the membrane in the same experim The venous outflow curve, which is the differentiated analogue of the residue curve all use of β-emitting and non radioactive indicators. The four calculations of the extract fraction represents four independent estimates

From a tracer kinetic point of view the presented principles attempt to get separate i formation from subsystems inside the main system

Theoretical considerations

Residue detection method

Extraction from blood to tissue

When an indicator which is able to permeate the capillary membrane is injected int arterially as a bolus the molecules are divided into two fractions according to their mo ments during the passage of an organ or tissue. One fraction, called the extracted fractio consists of molecules which leave and reenter the blood one or more times during t transit The remaining molecules, denoted the transmitted fraction, constitute the compl mentary fraction which stays intravascularly during the transit Registration of the indicat residue is possible by external counting when a γ-emitting indicator is used. The vascl transit of the transmitted fraction is very fast as compared to the transit of the extract fraction which also distributes in the larger extravascular space This space functions in t initial phase as a sink for the indicator when this is a foreign substance For these reaso the residue curve has a steep early downslope the formation of which is dominated by t fast vascular transit of the transmitted fraction through the relatively small intravascul volume, followed by a curve part with decreasing rate constant which is dominated by t slow transit of the extracted fraction *via* the relatively greater extravascular space.

The great difference in mean transit times for the two fractions of the indicator makes possible to resolve with good approximation the recorded residue curve in the individ transit curves for the transmitted and the extracted fraction.

The assumptions of the kinetic analysis are

1 Conservation of matter *i.e* the indicator substance in question is maintained ur altered inside the system.

2. Stationarity of the system, *i.e.*, the system is assumed to be in steady state with respe to blood flow distribution of transit times and volumes of distribution, and permeabili

3 Linearity of the system, *i.e* linearity (proportionality) between input and output fo different sizes of input.

4 Equivalent entry *i.e.*, the indicator particles are completely mixed with the inflowin blood (plasma) at the entrance to the system

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The mean transit time, $\bar{t}$, for an indicator through a system is the mean value of transit times for the indicator particles

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where t_1, t_2, t_3, ..., t_n denote the transit times of each particle, and n is the total number of particles

For any system, or subsystem which is only traversed once by the particles the m transit time can be determined as the area, A, divided by the height, H, of the residue cu (Zierler 1965)

$$\bar{t} = \frac{A}{H}$$

The mean sojourn time, θ is the mean occupancy time of indicator particles withi system or subsystem placed inside the main system. For the total system $\bar{t} = \theta$ but fo subsystem θ can be greater than $\bar{t}$ if the indicator particles traverses the subsystem more t once.

As determined by residue detection the equation for the mean sojourn time, θ in a sys or subsystem is analogous to equation 3

$$\theta = \frac{A}{H}$$

where A and H is area and height of the curve for system or subsystem.

In the present experiments the mean transit time of the indicator for the whole sys $\bar{t}$(total), is the sum of the mean transit time through the subsystem of the vascular vol of distribution, $\bar{t}$(iv), and the mean sojourn time in the subsystem, the extravascular vol of distribution, θ(ev), times the extracted fraction E. All indicator particles traverse vascular volume of distribution only once due to domination of convective transport in subsystem, but in the extravascular volume of distribution there is a possibility for m than one traversal of some indicator particles. The total amount of indicator traverses vascular volume of distribution but only the fraction E of this permeates into the ex vascular volume of distribution. This leads to the following equation

$$1\ \bar{t}(\text{total}) = 1\ \bar{t}(\text{iv}) + E\ \theta(\text{ev})$$

where 1 is the total amount of indicator given as a fraction $(1 = T + E)$

If the curve representing the transit of the extracted fraction, in the following called extravascular curve, is to be described by more than one exponential function, E is n constant during the washout process. But it is possible to obtain an average value c denoted $\bar{E}$(res) for the whole washout process from tissue to blood measured by resi detection In the following expression equation (5) is solved for E, and $\bar{E}$(res) is introdu

$$\bar{E}(\text{res}) = \frac{\bar{t}(\text{total}) - \bar{t}(\text{iv})}{\theta(\text{ev})}$$

Venous sampling method

Extraction from blood to tissue

From the venous outflow function it is possible to calculate the extraction from bloo tissue, by a curve extrapolation analogous to that described for the residue detection t nique. It is by the venous sampling technique also possible to use β-emitting and non-ra active indicators.

Fulfilment of assumptions number 1 2, 3 4 6, and 9 are also needed for the venous sampling technique. As area under a venous curve represents the same as height on the residue curve, the extraction fraction, E(vein), can be calculated in analogy with equation (1):

$$E(\text{vein}) = \frac{A(\text{ev})}{A(\text{total})} \qquad (7)$$

where A(ev) and A(total) are the areas under the extravascular and the total venous concentration curves, respectively

Extraction from tissue to blood

On the basis of assumptions number 1 2, 3, 4, 6, and 9 and analogous to the transformation of area on the venous curve to height on the residue curve and time weighted area on the venous curve to area on the residue curve, the venous sampling technique allows calculation of the average extraction, $\bar{E}$(vein), also for the transcapillary passage in the direction from tissue to blood. The time weighted area is defined as the sum of the products of time and counting activity (or concentration) for each sample. Transforming the ratio area over height for the residue curve, A(res)/H(res), to the ratio time weighted area, Aw over area, A, for the venous concentration curve, Aw(vein)/A(vein), in calculation of the mean transit times and the mean sojourn time then the right side of the equation becomes the same as in equation (6)

$$\bar{E}(\text{vein}) \frac{\bar{t}(\text{total}) \quad \bar{t}(\text{rv})}{\bar{\theta}(\text{ev})} \qquad (8)$$

In estimation of mean transit times and sojourn time the catheter effect must be corrected for in the venous sampling technique. This can be performed by simple subtraction of the mean transit time for the catheter from all three terms on the right side in equation (8). This means minus and plus the same value in the nominator and it cancels out. Thus only the denominator has to be corrected in this calculation. If the volume of the cather and the outflow from the system are measured the mean transit time for the catheter $\bar{t}$(cath), can be calculated (Zierler 1965).

$$\bar{t}(\text{cath}) = \frac{V(\text{cath})}{F} \qquad (9)$$

where, $\bar{t}$(cath), is the mean transit time of the catheter V(cath), the catheter volume, and F the blood flow In analogy with equation (9) blood flow through the vascular volume can be calculated from the mean transit time of the intravascular transit of an indicator and the vascular distribution volume. This general equation makes it also possible to calculate volumes of distribution from mean transit time and flow For the subsystem the extravascular volume of distribution the $\bar{\theta}$ is used instead of $\bar{t}$ in the calculation.

The final monoexponential component can be taken to represent washout from a well mixed biological compartment.

The indicator diffusion method (Chinard et al. 1955 and Crone 1963) employed in this comparative study has the following assumptions number 1 2, 3 4, and 6 mentioned above and in addition.

For any system, or subsystem, which is only traversed once by the particles the m transit time can be determined as the area, A divided by the height, H, of the residue cr (Zierler 1965)

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From the venous outflow function it is possible to calculate the extraction from bloo tissue, by a curve extrapolation analogous to that described for the residue detection te nique. It is by the venous sampling technique also possible to use β-emitting and non-ra active indicators.

corrections for values of blinds of plasma were performed. The Chromium-51 or Iodine-131 activity spectate and samples were counted in well-type scintillation detector (NaI (Tl) 2 inches diameter) ated around the maximum γ-peak of the respective radioisotopes. Counting was continued until about 0 counts or more had been recorded.

The blood pressure was continuously recorded by Statham (p 23 AA) transducer with saline filled ter placed in the contralateral femoral artery.

Bolus injections of Chromium-51-EDTA (ethylene-diamine-tetra-acetic-acid) or Iodine-131-thalamat T_{1824} prebound to serum albumin mixed in isotonic saline were performed within about 1 second. volume of injection was 0.1 ml. Immediately after the injection the contents in the catheter was with- by the injection syringe.

Calculations

Computations and calculations were performed in accordance with the procedures described above.

The peak heights of the measured residue curves was calculated as an average of the 4 to 6 highest counts count figures.

Extrapolation to peak time of the registered curve, as measured after the end of the time interval of the vascular transit, was performed either by simulating the curve part by the sum of three exponentials or monoexponential curve fitting of the early part of the curve from 1 to 3 minutes after the bolus injection. The last mentioned procedure was performed as compromise between minimum effect of the ing of the curve and reasonable number of observations on which regression analyses could be formed. In the interval before peak time the extrapolation was performed by multiplying the measured ues by the ratio between the height of the extrapolated curve at peak time and the peak value of the sured curve. For the peak plasma the same calculation procedure was used for this interval. The tribution of the measured values on the upstroke part of the curve was done in order to calculate the al areas under the curves. For the same reason it was necessary to extrapolate the curve to infinity. This performed by monoexponential function with the rate constant of the later part of the registered rve (Lassen and Sejrsen 1971). This extrapolation procedure was used both on residue and venous outflow rves. The area under the tail part of the residue curve, A(tail, res), extrapolated to infinity was calculated m the half-time ($T_{\frac{1}{2}}$) of the monoexponential down slope of the later part of the curve by the formula

$$A(\text{tail, res}) = H(\text{tail}) \cdot \frac{T_{\frac{1}{2}}}{\ln 2} \tag{10}$$

where H(tail) is the height of the tail part at the end of the experiment, and ln 2 is the natural logarithm 2.

The area under the venous curve A(tail, ven), was obtained in similar way:

$$A(\text{tail, ven}) \quad H(\text{tail}) \quad \frac{T_{\frac{1}{2}}}{\ln} \tag{11}$$

The time weighted area under the venous curve, Aw(tail, ven), was calculated by the formula:

$$Aw(\text{tail, ven}) \quad H(\text{tail}) \quad \frac{T_{\frac{1}{2}}}{\ln 2} \left(\frac{T_{\frac{1}{2}}}{\ln 2} \quad \right) \tag{12}$$

here the time at the end of the experiment.

In analogy with equation (9) plasmaflow was computed using the extravascular mean transit time, $\bar{t}(rv)$, obtained by retropolation and subtraction, and value for the vascular plasma volume obtained in parate experiments. Furthermore, the plasma volume was calculated from the $\bar{t}(rv)$ measured for the equilibrium curves using the same equation and the directly measured blood flow and the hematocrit ation.

In accordance with the single injection, venous sampling technique (Chinard *et al.* 1955, Crone 1963), calculation of the capillary extraction fraction (E) was performed by the formula.

$$E \quad 1 \quad \frac{\text{relative concentration of test substance in sample}}{\text{relative concentration of intravascular indicator in sample}} \tag{13}$$

The concentrations in the samples are here given relative to the concentration in the injectate. Calculation of an average extraction value was performed by taking the ratio between the area under the test substance curve until the time where the reference curve was reduced to 40 per cent of maximum and the area under the curve of the reference substance in the same time interval. This average ratio was then inserted in equation (13) instead of the ratio of the relative concentrations of the two substances in one sample.

10 The reference molecules must be representative for the vascular transit of the t molecules, *i.e.* equal Taylor diffusion conditions for the two molecules (Lassen and C 1970)

11 Back diffusion of indicator from the extravascular space to the blood must be negig in the initial phase, *i.e.* the extravascular space must function as a sink for the indic when this is a foreign substance

Experimental

Isolated autoperfused gastrocnemius preparation

The experiments were performed on 7 cat weighing from 3 0 to 4 5 kg. After ether induction chlor (70 mg/kg) was given intrav nously Tracheotomy was performed, nd the rectal temperature of the was kept at 37°C by heati g lamps controlled by a thermostat. Heparin (5 mg/kg) as given intraven

The skin of the right leg down to the paw was removed by thermocautery The tendo calcaneus wa close to calcaneus, and the gast ocnemius muscle was isolated by blunt dissection. The thigh muscles d vided just proximal to the knee joint nd crus was removed by disarticulation. The cavity of the fe bone w s plugged by cotton soaked in vaseline and the periosteal membrane was removed. The fe artery and vein were isolated at the lowe 2/3 of the thigh, and all side branches were ligated, except one distal branch of the artery in which a fine polyethylene catheter was inserted until the tip was at level of the wall of the femoral artery The femoral vein was cut and cannulated with a 1.5 cm long thylene t be (i.d 1.5 mm) connected to a 16 cm long glass t be (i d. 2.8 mm) used as enous out nd placed in level with the muscle The muscle was wrapped in moist gauze and covered by a p ethylene sheet which was kept i place during the preparatio and throughout the e periment. The temp ture of the muscle w kept at 37°C by a heati g lamp. The muscle was suspended on a stand ith a le of the muscle equal to the normal resti g length. Different blood flow levels, but fairly high and con in each experime t, were obtained by electrical stimulation of the isolated sciatic nerve by silver electr using a S4 Grass stimulator The frequency was from 1 per 3 seconds to per second, the oltage 7 V the duration of the square pulses w s 1 msec Blood flow was measured d rectly as venous outflow i a graduated cylinder nd a stop watch, several times before and d ring each experiment. Hematocrit w were measured n these blood samples. To avoid recirculation the venous blood from the isolated gast nemius muscle was vasted and the cat was given donor blood from 2 to 5 cats. This amount of do blood was sufficient to give the experiments a duration of about 2 hours.

The carefully shielded scint llation detector a N I (Tl) crystal with diameter of 1 1/4 inch as situ 10 cm medially t the muscle nd collimated to see only muscle tissue proper The pulses from scintillation detector were fed int scaler printer (Med tronic, Denmark) which was adjusted t reg the γ-emission round the ma mum energy peaks of Chromium-51 o Iod ne-131 The count ates printed out every half r one second in the initial 60 seconds period and every minute in the follo one to two hours.

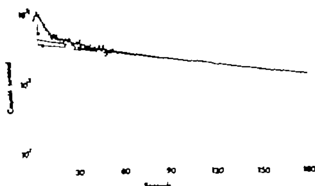
I order t reduce the background acti ty the whole muscle preparation was surrounded by lead b which resulted i stabl background activity of about 2 cou ts per second.

At the end of each e periment the femoral artery and vei were clamped and the nerve stimuli stopped. The counting level (a) was registered in i tervals from 15 to 30 min. The polyethylene and g covering the muscle were removed and the muscle surface was gently washed w th soap and ater t move the small amount of tracer deposited on th surface In nother 15 to 30 minutes period the cou level (b) wa measured under the same cond tions. A third counting period ( ) of the same length follo after separate removal of the muscle Correction f background activity and the act vity deposited on surface of the muscle and i the gauze was performed as a subtraction of the activity lev l. a–(b–c) resu in a corrected curve with a final activity equal to that of the muscle tissue separately

After the experiment the weights of both gastrocnemius muscles were measured. A eight differ was taken as weight change of the test muscl d ring the periment.

Venous sampling was performed every 2 second during the initial 60 seconds nd every fifth m during the following 1 to 2 hours. The samples (0.1 to 0.5 ml) were t ken in small glass vials. Plasma sa were obtained by ce trifugation.

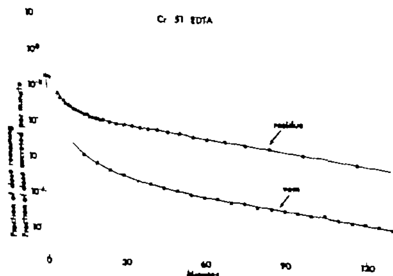
The T_{1824} concentrations in i jectate and venous plasma samples were estimated by a Beckma spectrophotometer at 620 m.



1 Curve measured after bolus [illegible]ction of Cr 51-EDTA and re[illegible] detection. Registration of [illegible] 3 [illegible] [illegible]th 3-exponen[illegible] and monoexponential retro[illegible]tion to peak time of the extra[illegible] [illegible]lar curv[illegible] part. The triangles [illegible]ote the intravascular curve [illegible] obtained by subtraction of the [illegible]es for the 3-exponential curve [illegible] the registered curve

[illegible]ble I lists experimental data from the 7 expts. Muscle weight, the weight excess of the [illegible] muscle compared to the contralateral muscle, and hematocrit values in per cent. Further[illegible]e, this table shows plasma flow calculated from the directly measured venous outflow [illegible]e initial phase and as average value $\bar{x} \pm SE$ for the whole experiment, and plasma flows [illegible]ulated from the intravascular transit curve obtained by residue detection and venous [illegible]pling. In these calculations a value for the intravascular volume of distribution of 3.0 [illegible] cent of the tissue volume was used (see supplementary experiments denoted 4), and [illegible]w). Average values $\bar{~} \pm SE$ for experiment number 1 to 6 or 3 to 6 are listed below for [illegible] four last mentioned parameters.

[illegible]able II presents the extraction fraction as estimated by 3 independent calculations in [illegible] first 3 experiments and by 5 in expt. number 4 to 7. No significant differences were [illegible]nd between the extraction values obtained by the five methods. Cr 51 EDTA was used



[illegible]ig. 2. Curves measured after bolus injection of Cr 51-EDTA with residue and venous outflow detection [illegible]caled to the height 1 for the residue curve and the area 1 for the venous curve.

Calculation of the capillary diffusion capacity (CDC) was performed from the equation.

$$CDC = -f_{pl} \cdot 0.89 \cdot \ln(1-E) \text{ (ml/100 g min)}$$

where f_{pl} is flow of plasma per 100 g tissue ln is the natural logarithm, and 0.89 is a factor correcting the concentration differences in plasma and interstitial fluid at equilibrium (Lassen and Trap-Jensen taking into account both the difference in water space in the two phases and the effect of the transendothelial potential difference for negatively charged, monovalent ions.

Supplementary experiment

Special experiments were performed to study 1) the input function, 2) the fastest transit through the system measured by the residue detection or venous sampling without catheter 3) the response curve for venous catheter and 4) the vascular volume of distribution in the muscle.

1) The input function to the autoperfused gastrocnemius preparation was studied by cutting the femoral artery at the outer zone of the external counting field After cutting the artery the blood flow was adjusted by a screw clamp and kept constant at levels used in the other experiments. The bolus injection technique described above was used for a bolus of Cr 51 labelled erythrocytes. When the blood was leaving the artery it passed the counting field falling through the air and it was collected in a glass vial situated outside the counting field. By this technique the output function from the artery to the counting field was obtained, *i.e.* the input curve to the muscle

2) The fastest transit through the system was studied by cutting the femoral vein at the outer zone of the external counting field where sampling was performed directly in small glass vials. Cr 51 labelled erythrocytes were used as indicator In this series of experiments as in the following the bolus injection technique and blood flow at the levels of the main series of experiments were used

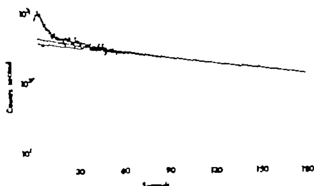
3) Bolus response curves for the catheter alone were obtained during perfusion from a blood reservoir by hydrostatic pressure force In these experiments the temperature of the blood in the reservoir and the tube leading to the catheter and the catheter was kept at 37°C. A bolus of Cr 51 labelled erythrocytes was injected directly into the initial part of the inflow end of the venous sampling catheter Outflow samples were taken as in the main experiments.

4) Vascular volume of distribution was determined using the intravascular tracers T_{1824}-albumin and Cr 51 labelled erythrocytes. The mean transit time of these tracers multiplied by the directly measured venous outflow of blood yielded the intravascular volume for the tracers in analogy with equation (and by using the hematocrit value the intravascular plasma volume was computed.

Results

Chromium-51 EDTA was used as test substance in six experiments and Iodine 131-iodipamate in one. T_{1824}-albumin was used as an intravascular indicator in all expts. Representative residue curves plotted in semilogarithmic scale are shown in Fig. 1 and 2 with different time axis In Fig. 1 is shown the initial 3 min of the residue curve with a fitting of the curve after the first minute by the sum of three exponentials and extrapolation back to peak time as described above. The sum of three exponentials fitted the curves very well in the time interval 1 to 130 minutes after the bolus injection as seen in Fig. 2, where the line drawn through the dots is the sum of three exponentials. Also a monoexponential retropolation with a curve fitting from 60 to 180 seconds is shown in Fig. 1 the lower line. In Fig. 2 the residue curve is scaled to peak height equal to one and the simultaneously measured venous curve also presented in this figure is scaled to area under the curve equal to one.

The bolus injection venous sampling method using vascular reference indicator is presented in Fig. 3 and 4 The concentrations of test and reference indicator in samples relative to concentration in injectate are plotted linearly as a function of time in Fig. 3 For the same experiment the extraction values calculated from equation (13) are plotted versus time in Fig. 4



1 Curv measured after bolus ...tion of Cr 51 EDTA and re... detection Registration of ...nal 3 min ...th 3-exponen... and monoexponential retro... tion to peak time of the extra... ular curve part. The triangles ...es the intravascular curve obtained by subtraction of the ...a for the 3-exponential curve the registered curve.

Table I lists experimental data from the 7 expts. Muscle weight, the weight excess of the ...t muscle compared to the contralateral muscle, and hematocrit values in per cent. Further...re, this table shows plasma flows calculated from the directly measured venous outflow ... the initial phase and as average value ± SE for the whole experiment, and plasma flows ...culated from the intravascular transit curve obtained by residue detection and venous ...mpling I these calculations a value for the intravascular volume of distribution of 3.0 ... cent of the tissue volume was used (see supplementary experiments denoted 4), and ...low) A...erage values $\bar{x}$ ± SE for experiment number 1 to 6 or 3 to 6 are listed below for ...e four last mentioned parameters.

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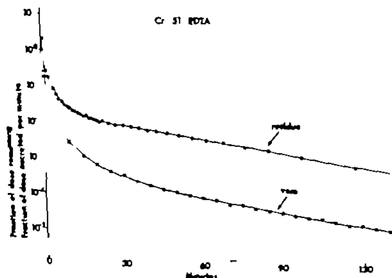


Fig. 2. Curves measured after bolus injection of Cr-51-EDTA with residue and venous outflow detection scaled to the height 1 for the residue curve and the area 1 for the venous curve.

Calculation of the capillary diffusion capacity (CDC) was performed from the equation:

$$CDC = -f_{pl}\ 0.89\ \ln(1-E)\ (ml/100\ g\ min)$$

where f_{pl} is flow of plasma per 100 g tissue. ln is the natural logarithm, and 0.89 is a factor correcting the concentration differences in plasma and interstitial fluid at equilibrium (Lassen and Trap-Jensen ...) taking into account both the difference in water space in the two phases and the effect of the transmembrane potential difference for negatively charged, monovalent ions.

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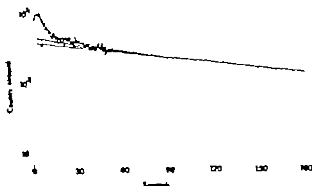
3) Bolus response curves for the catheter alone were obtained during perfusion from a blood reservoir by hydrostatic pressure force. In these experiments the temperature of the blood in the reservoir, the tube leading to the catheter and the catheter was kept at 37°C. A bolus of Cr 51 labelled erythrocytes was injected directly into the initial part of the inflow end of the venous sampling catheter. Outflow samples were taken as in the main experiments.

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1 Curv. measured after bolus ...ion of Cr 51 EDTA and re... detection. Registration of ...total 3 min ...th 3-exponen... and monoexponential retro... ...n to peak time of the extra... ...lar curve part. The triangles ...tes the intravascular curve obtained by subtraction of the ...s for the 3-exponential curve ... the registered curve.

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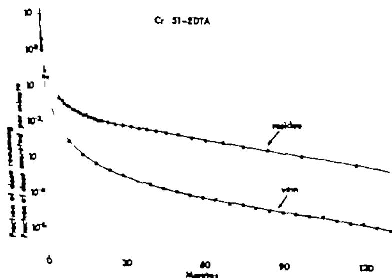


Fig. 2. Curves measured after bolus injection of Cr-51-EDTA with residue and venous outflow detection ...ttled to the height 1 for the residue curve and the area 1 for the venous curve.

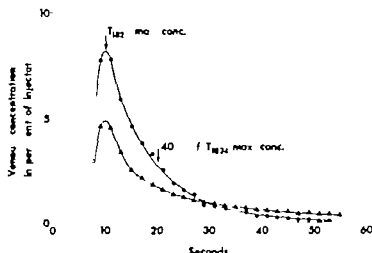


Fig. 3. Washout curves after injection of T_{1824}-albumin and Cr-51 EDTA registered by venous sampling. Dots and triangles denotes the T_{1824}-albumin and Cr-51 EDTA values given in per cent of the injectate.

as indicator in expt number 1 to 6 and I 131 thalamate in expt number 7 The capillary diffusion capacity CDC was calculated from directly measured plasma flows, mean values $\bar{x} \pm$ SE for expt number 1 to 6 and 4 to 6 are listed below Furthermore, CDC values calculated from plasma flows obtained by curve resolution for the four methods are presented with average values $\pm$ SE for expt. number 1 to 6 and 4 to 6. No significant differences were found between the CDC values obtained by the five independent methods.

Monoexponential retropolation to peak time of the extravascular transit curve in the interval from 60 to 180 s was found to underestimate the extraction with 11.8 per cent in average compared to the 3 exponential curve resolution. Also the estimate of the extravascular mean transit time was different with the two extrapolations. Monoexponential analysis gave an overestimation of this value by 9.5 per cent which in part counterbalanced the underestimation of the E value at the calculation of the CDC value. The combined effect on the CDC value is in average a 3.2 per cent underestimation by the monoexponential extrapolation as compared to the three exponential extrapolation

The extravascular curves showed a bended shape with in average a factor of 12.8 between the rate constant of the beginning and the final curve part.

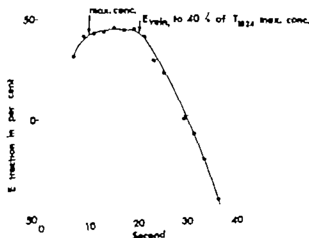


Fig 4 E fraction values calculated from values of T_{1824}-albumin and Cr 51-EDTA in each sample.

ᴸᴱ I. Muscle weight, edema, hematocrit, initial and average plasma flows. For expt. no. 1 to 6 and 4 to 6, average ± SE are given below

x	Muscle weight (g)	Muscle edema (per cent)	Hematocrit (per cent)	Plasma flow initial phase (ml/100 g·min)			Plasma flow average values (ml/100 g min)	
				Calculated from directly measured blood flow	Calculated from residue curve	Calculated from venous curve	Calculated from directly measured blood flow	SE
	19.6	7.1	37.4	15.8	19.4		14.0	0.29
	18.7	12.6	34.6	23.3	24.5		21.7	0.45
	11.6	1.6	30.6	12.1	17.8		12.3	0.04
	42.4	0.2	38.4	11.9	9.8	9.7	10.2	0.27
	29.5	10.1	34.8	13.7	17.3	16.3	11.7	0.23
	18.8	4.3	34.0	11.6	11.8	9.9	11.4	0.07
	27.8	17.3	33.5	18.4	17.4	22.9	15.8	0.20
				6	6	3	6	
				14.7	16.8	1 0	13.9	
SE				±1.8	±2.2	±2.2	±2.0	

Data from expt. number 4 to 7 have been presented in a previous article concerning monoexponential extrapolation of the final slowest part of washout curves (Larsen and ...gren 1971). The corresponding numbers of the expts. in that article are 1, 3, 5 and 7. In ...ch of these expts. there was a close agreement between the rate constants calculated from ...e final curve parts of the residue and venous curves. Three experiments from the quoted ...ticle were excluded from the material presented in this article: two (no. 4 and 6) due to ...ck of agreement between these two final rate constants within the single expts., and one ...o. 4) due to a delayed bolus input.

Table III shows the volumes of distribution for the indicators as calculated from the ...sidue and venous curves in the total system, in the intravascular part, in the interstitial ...rt and in the part defined by the monoexponential tail of the curve. Average values ± SE ...r expts. number 1 to 7 and 4 to 7 are listed below.

Calculation of the intravascular plasma volume from the T_{131}-albumin curves with ...orrection for the effect of the catheter gave in average ± SE, 1.9 ± 0.1 ml/100 g of tissue ...7), which is in agreement with the results obtained by kinetic analysis.

The results of the supplementary experiments were the following:

1) The input function to the muscle had a duration of about 1 s during which 99 per cent of the indicator had arrived (2 expts.) at plasma flows used in the main series. The input ...nction can also be evaluated from the increasing part of the residue curves.

2) The shortest transit times determined by venous sampling were from 3 to 6 s at plasma ...low values as those in the main series (4 expts.). The venous curves for intravascular in...dicators could be simulated by the sum of two exponentials with a factor of about 2 to 5 between the rate constants of the fast and the slow component. The interception with the ...ordinate were about 95 per cent of the venous curve height for the fast and 5 per cent for the ...slow component.

TABLE II. Capillary extraction fraction measured simultaneously by 5 independent methods with corresponding values for capillary diffusion capacity For expt. no. 1 to 6 or 4 to 6, average are given below The CDC values are given in ml/100 g min.

Expt no	Indicator	E (T_{1824})	E (res.)	Ē (res.)	E (vein)	Ē (ve
1	Cr 51 EDTA	0.293	0.249	0.233		
2	Cr 51 EDTA	0.203	0.156	0.148		
3	Cr 51 EDTA	0.281	0 304	0.298		
4	Cr 51 EDTA	0.430	0.373	0.350	0.398	[illegible]
5	Cr 51 EDTA	0.297	0.290	0.283	0.273	[illegible]
6	Cr 51 EDTA	0.432	0.448	0.438	0.450	[illegible]
7	I 131-thalamate	0.287	0.273	0.269	0.306	[illegible]
			CDC calculated from plasma flows obtained by curve resolution			
1	Cr 51 EDTA		4 91	4.06		
2	Cr 51 EDTA		3 69	3.55		
3	Cr 51 EDTA		5 69	5.69		
4	Cr 51 EDTA		4 07	3.22	4.38	[illegible]
5	Cr 51 EDTA		5 15	4.37	4 63	[illegible]
6	Cr 51 EDTA		6..4	5 95	5.27	[illegible]
7	I 131-thalamat		4 94	4 17	7 44	[illegible]
n			6	6	3	3
x			4 96	4 47	4 76	[illegible]
±SE			±0.39	±0.46	±0.26	±[illegible]
		CDC calculated from directly measured plasma flows				
1	C 51 EDTA	4.88	4 03	3 31		
2	Cr 51 EDTA	4 71	3.52	3.38		
3	Cr 51 EDTA	3.55	3 90	3.87		
4	Cr 51 EDTA	5.95	4.94	3.91	5.37	[illegible]
5	Cr 51 EDTA	4.30	4 18	3 46	3.89	[illegible]
6	Cr 51 EDTA	5.84	6.13	5.85	6.17	[illegible]
7	I 131 thalamat	5.54	5.22	4 41	5 98	[illegible]
n		6	6	6	3	3
		4.87	4 45	3 96	5 14	[illegible]
±SE		±0.47	±0.39	±0.39	±0.67	±[illegible]

3) The bolus input expts. performed with the venous catheter alone showed similar bended curve shapes, and the transit time for the whole curve was equal to the volume blood flow ratio Convolution of the intravascular curve part obtained from residue curve with the catheter response curves obtained at equal blood flow values yielded curve shape equal to the venous outflow curves for intravascular indicators. Deconvolution of T-albumin curves with the cather response curves gave curve shapes almost identical to the intravascular curve part of the residue curves Comparison of T_{1824}-albumin curves with the intravascular curve part of venous curves showed almost identical shapes of these curve These comparisons of curve shapes were used as indirect tests of the reliability of the extrapolation procedures employed in the main series of expts.

4) The vascular volume of distribution was determined in 6 expts. with Cr 51 labelled erythrocytes and in 2 expts. with T -albumin as indicator The intravascular volume

LE III Volumes of distribution for Cr-51-EDTA and I-131-thalamate calculated from residue and out flow curves. Average ± SE is given below for 7 and 4 expts.

	Total volume of distribution (ml/100 g)		Intravascular volume of distribution (ml/100 g)		Extravascular volume of distribution (ml/100 g)		"Final" volume of distribution** (ml/100 g)	
	Res	Vein	Res.	Vein	Res.	Vein	Res.	Vein
	10 6		1 6		9.0		2.9	
	9 0		1 9		7 1		2.4	
	15 9		1.5		14 4		3 8	
	11 3	11 9	2.3	2 3	9 0	9 6	4.8	5.3
	16 4	17 1	1.5	1.6	14.9	15.5	5.9	5.8
	19 7	20 2	1.9	2 3	17.8	17.9	6.8	7.3
	16 7	19 4	2.2	1 6	14 5	17.8	9 1	9 7
	7	4	7	4	7	4	7	4
	14.2	17.2	1 8	2.0	12.4	15.2	5.4	7.0
IE	±1.5	±1.9	±0 1	±0.2	±1.5	±1.9	±0 9	±1.0

the intravascular plasma volume.
the volume of distribution defined by the final washout rate.

tained by the two indicators gave for all 8 expts. expressed in per cent of muscle weight average value ± SE. 3.0 ± 0 4 ml/100 g. As mentioned previously this value was used in e calculation of plasma flow from the intravascular curve parts.

Discussion

ethodological considerations

e fulfilment of the assumptions mentioned in theoretical considerations is considered in e following:

1 The indicator Cr 51 EDTA is inert. Thus conservation of matter is fulfilled for this dicator This is presumably also valid for I 131-thalamate.

2 Stationarity of blood flow was evaluated from the directly measured blood flow Only inor differences were observed between the initial and the average values, indicating a inimum perturbation effect of the injection. The standard error of the average plasma flows as from about 2 to 10 per cent, which is considered an acceptable approximation to sta- onarity of flow during the expts.

3 Linearity of the system is probable as the indicators used were inert and on this basis e response of the system should be proportional to the input. No observations seem to dicate non-linearity of the system.

4. Equivalent entry is presumably obtained by the fast injection of the bolus in a direc on transversal to the blood stream.

5 Isoefficiency counting of the indicator particles in the different volumes of distribu- on must be obtained as the tissue elements (cells, interstitial space and capillaries) are qually mixed in all tissue regions. However there is as mentioned no demand of isoefficient counting from one region to another in the tissue.

6 Recirculation was completely eliminated by the experimental technique.

7 The measurement of the height of the registered curve was examined from the expe ments where the duration of the input and the shortest transit time of the system were me ured at different blood flow values. The analysis indicates that a time interval of 2 to exists in which measurement of the correct height of the curve is possible at the blo flow values actually used.

8 Considering the relatively great diffusion velocity for the indicator in water and relatively short diffusion distances in a considerable part of the interstitial space situ in close relation to the capillaries, it is reasonable to expect that the indicator is relat well mixed in this "subcompartment Consequently it can be expected that the initial ph of the washout of the extracted fraction will be dominated by this washout process in minutes yielding a possibility for a relatively exact extrapolation by a monoexpone function

The extrapolation of the extravascular curve part back to peak time was evaluated expe mentally by comparison of plasma flow values measured directly in the initial phase plasma flow values calculated from the intravascular volume of distribution and the me transit time of the intravascular curve part obtained by extrapolation and subtracti These results show no significant difference. These results are not incompatible with statement that the extrapolation procedure employed has been correct. Also a compari of the curve configurations was performed The curves were dominated by a mono ponential washout function over more than 95 per cent and they showed good agreem which is taken as a substantial support for the reliability of the extrapolation procedu employed It is important to note that all indicator molecules which pass the intersti space during their transit of the muscle will be considerably delayed compared to molecul remaining in the vascular space during the transit In average this delay was a decrease transport velocity of a factor 26 for molecules permeating the capillary membrane, and ev the fastest extravascular molecules had a washout rate which was at least a factor 7 slow than the slowest intravascular washout rate. This is good conditions for a separation of t two fractions by curve resolution

The results show that the CDC values are rather insensible of the extrapolation procedu used as both the extraction and the intravascular mean transit time are changed at differe extrapolation procedures which reduces the combined effect to a minimum On this ba the CDC values calculated from plasma flow obtained by curve resolution presumably to be preferred for those calculated from the directly measured flow

9 The total area under the curves was obtained by monoexponential extrapolation infinity The procedure was examined in a previous article (Lassen and Sejrsen 1971). Th final slopes were not significantly different by the two measurements within a single expe ment, indicating that the final slowest washout rate was obtained. This yields the pos bility to extrapolate correctly to infinity (Lassen and Sejrsen 1971)

10 The different 'Taylor diffusion effect for test and reference molecules was taken in account by the area calculation which tends to reduce this difference (Lassen and Cro 1970). This assumption is only of importance for the indicator diffusion method, as the te indicator serves as its own intravascular reference for the methods based on kinetic analys

1 Back diffusion of indicator from the extravascular space to the blood must be negligible . the initial phase to give the correct extraction value by the indicator diffusion method. ;pport for the fulfillment of this assumption has been taken from the existence of a hori-ntal part in the extraction curve (see Fig. 4). This horizontal curve part has been inter ted as the initial time interval, where back diffusion from tissue to blood presumably is gligible (Lassen and Crone 1970). Back diffusion is of no importance in the four kinetic thods as the retropolation procedures gives a description of this process and by this minate the effect at peak time.

From these considerations the assumptions of the methods seem to be reasonably ful-ed in the present experiments.

The extravascular curve part showed a bended shape which in average for all 7 expts. d factor of 12.8 between the rate constants of the early and the final part of this curve. this connection inhomogeneity of perfusion has to be considered. In previous studies with shout of Xenon-133 from local sites of the tissue, no signs of gross inhomogeneity of ood flow were found in the same muscle preparation at high and low blood flow values ejrsen and Tønnesen 1968 a, Tønnesen and Sejrsen 1970). Also by an intravascular indi-tor Iodide-131-albumin, no signs of a non uniform distribution of blood flow were ob-rved for the same muscle preparation (Sejrsen and Tønnesen 1968 b) In the blood perfused strocnemius muscle in dogs a rather homogeneous uptake of D_2O was demonstrated hompson *et al* 1959) which also indicates a rather homogeneous blood perfusion. In the esent study the intravascular curves were dominated by a monoexponential part consti-ting about 96.1 ± 1 7 per cent (average + SE, n 7) of the washout as obtained from $T_{...}$ lbumin curves. The rest can presumably be explained by the presence of small amounts of nnective tissue in the preparation and the parabolic shape of the flow profile in greater essels within the muscle preparation and the effect of the catheter *cf* the results obtained the experiments with perfusion of the catheter Thus rather high degree of homogeneity f blood flow in the capillaries and per gram of tissue mass seems to be evident from the resent observations and the grossly bended shape of the extravascular curve part cannot be xplained by inhomogeneity of blood flow The consequence of this is that the main assump-ons for the osmotic transient method (Pappenheimer *et al.* 1951) which is a single well ixed interstitial space obviously is not fulfilled as the extravascular curve part is not mono-xponential throughout its course.

Agreement was found between the permeability data obtained by the indicator diffusion nethod and the four methods based on kinetic analysis of the curves recorded by residue nd outflow detections after bolus injection. This can be interpreted as a mutual support or the methods. The "Taylor diffusion effect tends to give an overestimation of the extrac ion fraction as measured by the indicator diffusion method. The extrapolation procedures erformed when using the bolus injection, kinetic analysis methods were considered to ffect an overestimation of the extraction fraction. Theoretically a correction of the ex tracted fraction is needed as previously proposed (Sejrsen 1969 and Rakhile *et al.* 1976) for partial in series models. But the correction constitute only decrease of the E value of about 3 to 4 per cent and of the CDC value of about 1 per cent which is considered in-significant, and no correction has been performed. The diminutive difference between the

results obtained by the two types of methods indicates small or insignificant importan the "Taylor diffusion effect"

The agreement of extraction and CDC values found for the transport process in b directions over the capillary membrane supports the concept that the capillary memb functions as a symmetrical membrane for transport of small hydrophilic indicators. T finding is supported by the results of washout of Cr 51 EDTA after local injection in muscle in man compared to results obtained by the indicator diffusion method (Trap-Je Korsgaard and Lassen 1970, Trap-Jensen and Lassen 1971).

Capillary permeability coefficients

Calculation of the permeability coefficient P_d, (cm/s) for Cr 51 EDTA from the ave CDC values obtained by the indicator diffusion method and an overall average of the l other methods using calculated or directly measured plasmaflows, and a capillary su area of 7 000 cm /100 g of tissue (Pappenheimer *et al* 1951) gives P_d values of 1 16 10 0 11 10 cm/s (n 6) 1 10 10^{-5} ±0 05 10^{-5} cm/s (n = 18) and 1.05 10^{-5} ±0.06 10^{-5} c (n = 18), respectively The permeability calculated from the tracer kinetic methods for passage from blood to tissue gives 1 16 10 ±0 07 10^{-5} cm/s (n = 9) and from tissu blood 1 03 10 ±0 08 10^{-5} cm/s (n = 9) These permeability values are in agreement data obtained with the same preparation and method by Paaske (1977 a), who found value of 1 02 10 cm/s at a plasma flow of about 15 ml/100 g min

Crone (1963) used the indicator diffusion method for determination of the permea of sucrose in dog hind limbs which were considered predominantly to consist of m tissue Autoperfusion was performed at blood flows estimated to about 7 ml/100 g but this parameter was not measured A P_d value of 0 74 10^{-5} cm/s was obtained for su which has almost the same molecular weight as Cr 51 EDTA However the calculati the CDC value was performed from equation (14) using blood flow instead of plasma flow The concentration gradient over the capillary membrane in the initial phase was t to be equal to the logarithmic mean concentration in the capillary To represent the dr force for transcapillary diffusion the concentration has to be that in plasma water and sequently plasma water flow should be used for calculation. Correction for this gives value of about 0 37 10 cm/s This figure is about one half of those for muscle t proper at the plasma water flow level used (Paaske 1977 a) The discrepancy can b plained by the fact that the hind limb contains various tissues and furthermore shunt v in the paw

Rippe *et al* 1978 found by the indicator diffusion method on the rat hindquarter pre tion during artificial perfusion and maximum vasodilatation a CDC value for Cr E of 5 67 ml/100 g min which corresponds to a P_d value of 1.35 10^{-5} cm/s. This value little lower than the results obtained for the autoperfused cat gastrocnemius musc maximum exercise which was about 2.1 10 cm/s (Paaske 1977 a)

Another investigation of permeability of Cr 51 EDTA has been performed by the indi diffusion method in the exercising human forearm with an inflated cuff around the to avoid influence from tissues in the hand (Trap-Jensen and Lassen 1970 Trap-Je 1970, and Trap-Jensen and Lassen 1971) Blood samples were taken from a profound

...e cubital fossa. Also in this investigation the region under study was taken to consist ...ominantly of muscle tissue. P values of 0.77 10^{-5} to 0.88 10^{-5} cm/s were found at ...ma flows of 14 to 18.4 ml/100 g min. These results are closer to those obtained for the ...perfused cat gastrocnemius muscle at equal flow range. In this connection it is important ...ress that also in the mentioned two studies performed on rat hindquarter and human ...arm the relative amount of other tissue under study which is not muscle tissue, is ...siderably larger than in the gastrocnemius muscle preparation. Exercising skeletal muscle ...e has a higher capillary permeability than the other tissues included as shown by Paaske ...6, 1977 a, 1977 b, and 1977 c), Paaske and Nielsen (1976).

...n all calculations of P values a capillary surface area of 7 000 cm^2/100 g of tissue has ...n employed. As shown by Paaske (1977) recruitment of capillaries takes place with a ...tor of 4 at a plasma flow change from 2 to 30 ml/100 g min. The number of capillaries in ...ction per mm^2 is about 100 during rest and 400 during maximum exercise (Landis and ...ppenheimer 1963 Hammersen 1968, Eriksson and Myrhage 1972, Brodal *et al* 1977). ...culation of the capillary surface area from these figures and an average value of the ...illary diameter of 6.5 μm and the length of 1 200 μm gives a capillary surface area of ...out 2 500 and 10 000 cm^2/100 g of tissue, respectively At a plasma flow of 15 ml/100 ...min an area of 5 000 cm^2/100 g presumably is a better estimate to use, resulting in a correc...n of the P_d values to figures around 1.5 10^{-5} cm/s.

...he free diffusion coefficient in water D for Cr 51 EDTA is about 0.7 10^{-5} cm^2/s. From ...formula P = D/Δx, where Δx is "the membrane thickness" it is possible to express Δx ...the total path length across the membrane which becomes about 0.5 cm in 100 g of ...scle. As the capillary membrane is about 1 000 Å in thickness this means that the *effective* ...e area constitute about 1/50 000 of the capillary surface area.

...thodological possibilities

...bolus injection, residue or venous registration method for measurement of capillary ...meability using only a test substance (Sejrsen 1970) has been employed on other tissues ...h as brain (Eichling *et al* 1974 and Raichle *et al* 1976), cutaneous, subcutaneous, and ...eletal muscle tissue (Paaske 1976, 1977 a, b, c, and Paaske and Nielsen 1976) by residue ...ection and in a study of C 14-inulin permeability in skeletal muscle (Paaske and Sejrsen ...77) by venous detection. It is presumably possible to use even larger indicator molecules ...rhaps as large as albumin which make the method suitable for the study of the equivalent ...re size of the capillary membrane for transport of hydrophilic molecules, and the extra...scular volume of distribution for albumin. If the injected dose is chosen to be sufficiently ...gh, it is possible to measure and extrapolate the extravascular curve to peak time even at ...w extraction values The extracted fraction of the indicator will have a very slow wash...t rate in the direction from tissue to blood. This gives the possibility to determine the ...tivity level of the extracted fraction from a slowly falling curve which can be registered ...ver a long time interval, which compensates for the low counting value.

In contrast the indicator diffusion method has its limitation for indicators as large as ...ulin in skeletal muscle at a plasma flow level of 5 to 10 ml/100 g min, for which the relative ...oncentration difference between test and reference indicator becomes indistinct due to

the inaccuracy of both concentration measurements i.e. at low values of E. Thus it se[illegible] impossible to measure albumin extraction by this method even if labelled erythro[illegible] could be used as reference indicator

The bolus injection residue detection method is due to the simplicity of the method [illegible] for clinical purposes. Recirculation and background activity can often be measured o[illegible] symmetrical region and subtracted from the registered curve.

Volumes of distribution

The volumes of distribution determined by kinetic analysis for the hydrophilic indica[illegible] used are in accordance with results obtained in previous studies (Lassen and Sejrsen 19[illegible] Paaske and Sejrsen 1977). Difference in volume of distribution between sucrose and in[illegible] was found by Crone and Garlick (1970), but edema formation and insufficient tim[illegible] equilibration in these experiments can presumably explain these results. Kruhoffer (1946 [illegible] b) found also almost identical volumes of distribution for sucrose and inulin in neph[illegible] tomized rabbits.

The monoexponential final part of the washout curves constitute a considerable fract[illegible] almost 50 per cent, of the extravascular volume of distribution. For C 14-inulin the co[illegible] sponding value was found to be about 76 per cent (Paaske and Sejrsen 1977). In mu[illegible] tissue about one half of the interstitial space is located inside the longitudinal tubule[illegible] the sarcoplasmic reticulum (Porter and Palade 1957), which might be considered to acco[illegible] for the well mixed compartment responsible for the final monoexponential washout. T[illegible] sarcoplasmic reticulum consists of transversal and longitudinal tubules. Only longitudi[illegible] tubules have a limiting membrane to the other part of the interstitial space. The great[illegible] distance within the longitudinal tubules is about 2 μm and complete mixing by diffusion [illegible] obtained for the actual indicators over this short distance within a few ms. Thus the fi[illegible] monoexponential curve part could indicate the washout from the sarcoplasmic reticul[illegible] and then these final rate constants presumably is determined by the permeability of t[illegible] membrane of the lateral saccus in the longitudinal tubules. The total area of this membr[illegible] is from histometrical observations estimated to be about 6 times greater than the capill[illegible] membrane at a plasma flow of 15 ml/100 g min (Porter and Palade 1957). Calculation [illegible] the permeability from $E(tail) - \bar{t}(total)/\bar{t}(tail)$ leads to a sarcoplasmic diffusion capac[illegible] $SDC = -f_{p1}\ 0.89\ \ln(1-E) = 0.48$ ml/100 g min. This value is about 10 times lower th[illegible] that obtained for the capillary membrane at a plasma flow of 15 ml/100 g min. The pe[illegible] meability for the sarcoplasmic reticulum $P_d(SR)$ then becomes about $2.6\ 10^{-}$ cm/s wh[illegible] is about 60 times lower than the permeability of the capillary membrane at that flow le[illegible] and with a range from about 30 at rest to 120 at maximum exercise.

Autoradiography is suggested as a suitable method for examination of the validity of t[illegible] hypothesis for the final washout rate constant. Supported by such examinations this cur[illegible] analysis will constitute another attempt to gain information about a subsystem inside t[illegible] main system by use of kinetic analysis.

I want to acknowledge Sonja Kludt for excellent technical assistance.

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From the Department of Zoophysiology University of Göteborg, Sweden

Gastric acid secretion and water balance in the marine teleost *Gadus morhua*

By

Björn Holstein

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Abstract

HOLSTEIN, B. *Gastric acid secretion and water balance in the marine teleost Gadus morhua.* Acta physiol. scand. 1979 *105* 93–107

Gastric acid secretion was measured in unanaesthetized codfish surgically equipped with a catheter draining the stomach. The pylorus was ligated. Without perfusion of the intestine, or during perfusion with pure water (100 % SW) or slightly diluted (67 %) SW gastric acid secretion was low (< 10 μmol H⁺/kg h) the fishes became dehydrated during the experiment. Perfusion of the intestine with 50 % or 33 % SW enabled the fishes to compensate for the water loss to the environment and greatly enhanced gastric acid secretion. Acid secretion was elevated also in fishes in which the dehydration was prevented by an intra-muscular infusion of saline. Perfusion of the intestine (8.5 ml/h) decreased the hourly effluent volume ("drinking rate") from the stomach to almost zero within 2 h. The effect was independent of the degree of dilution of the perfusing SW but was less pronounced when the perfusion rate was lowered. Intra-muscular ... also depressed "drinking rate". The acid secretion prevailing during perfusion of the intestine with ... dilution preventing dehydration—suggested to represent the basal acid secretion of the codfish—inhibited by atropine, hexamethonium, and metiamide.

... marine fish looses water to the hyperosmotic environment, and to compensate for this, ... has to drink continuously (Smith 1930). This drinking was utilized in a technique developed ... this laboratory (Holstein 1975) for *in vivo* studies of the regulation of gastric acid secre... n in the Codfish. Under MS-222 anesthesia a polyethylene cannula was placed in the ... stomach so that the water swallowed by the fish, mixed with the gastric secretions, could ... collected. The method was subsequently modified to allow for correction of losses ... through the oesophagus (Holstein 1977). The surgical procedure included the ligation of ... pylorus.

As the uptake of water takes place in the intestine, it was suspected that the pylorus-ligated fishes might suffer from dehydration. This has been confirmed by weighing fishes ... the end of the experiments, i.e. on the third day post-surgically: the BW was then 87.9 ± ...5 % (mean and S.E. of 40 animals) of the presurgical value. This report describes a technique ... the *in vivo* perfusion of the intestine. It was found that the fishes could compensate for ... the water loss provided that the sea-water used for the perfusion was sufficiently diluted, ... and that this was accompanied by a profoundly increased secretion of acid from the stomach. ... Some observations on drinking rate are also reported.

Materials and Methods

Codfishes of both sexes, weighing between 290 and 730 g were used. Before use, the fishes were kept without food for at least one week in the aquarium (10°C). Surgery was carried out under MS-222 anesthesia as described earlier (Holstein 1975). The following catheters were positioned (cf. Fig. 1):

1. *Intestinal perfusion catheter* (C1). The catheter was made from polyethylene tubing (PE 50, Intramedic), its back end flared to prevent it from sliding through the intestinal wall. To aid the positioning, the leading end of the catheter was equipped with a 0.8 mm needle. Through an incision made a few mm proximal to the pyloric appendices, the needle was inserted into the gastro-intestinal lumen, guided past the appendices and taken through the intestinal wall (Fig. 1 A). The accompanying PE 50 was then pulled until the flange was stopped by the luminal surface. The catheter, previously permanently bent 90° by heating a few mm from the flange, was secured by a purse-string suture in the muscular coat of the intestine.

2. *Catheter draining the stomach* (C2). The catheter was made by flaring a piece of PE 200 tubing. A perspex sond was used to introduce the catheter into the stomach by way of the mouth. The procedure has been described in detail (Holstein 1975), but was slightly modified. Instead of taking the catheter through the intestinal wall, it was now exteriorized through the incision proximal to the pyloric appendices (Fig. 1 A, B). The ligatures L1 and L2 were then tied on either side of the incision.

An alternative technique to drain the stomach was suggested by Cederberg of this institute. A rigid cannula was made from PE 200 tubing by flaring and imposing a permanent bend 5 mm from the flange (C'2, Fig. 1 C). The cannula was positioned by a technique analogous to the one used for C1 (above), but a coarser needle (1.4 mm) and a piece of PE 200 aided the positioning. A tygon tubing was used to connect the cannula to a fraction collector.

3. *Catheter for perfusion of the stomach* (C3). Through the mouth a straight perspex sond was inserted into the stomach (Fig. 1 C). A piece of PE 50 tubing was attached to the blunt end of an injection needle and the needle inserted through the stomach wall, into the lumen and into the sond. The tubing was pushed until the needle was visible outside the mouth, the sond removed and C3—constructed as C1—attached to the needle. By retracting the PE 50 tubing the flange of C3 was positioned against the mucosal surface of the anterior part of the stomach. A purse-string suture secured the catheter in the muscular coat of the stomach. The catheters C1, C2 or C'2, and C3 were exteriorized through stab wounds in the body wall, and the body wall closed by a continuous suture. C2/C'2 was connected to a fraction collector for the outflow in one hour fractions, and C1 and C3 to roller pumps.

Experimental schedule. The surgery was carried out during the a.m. and following recovery from anesthesia the fishes were placed in separate tanks and the catheters connected as described. Perfusion of the intestine, when employed, started at once or, in some initial studies, on the morning of the second day. Unless otherwise stated, the perfusion rate was 8.5 ml/h (corresponding to "high rate" of ...). The perfusion fluid was either sea water (100% SW) or SW diluted with distilled water to 67 or 50% SW). Perfusion of the stomach started 20–22 h following surgery. The perfusion fluid was 100% SW containing 100 mg/l phenol red (PR), and was delivered at 8.5 ml/h.

In the experiments with secretory antagonists, these were administered during the second day. A Perfusor IV Infusion Pump was employed for the i.m. injection of the drugs (0.62 ml/h).

When interrupting the experiments, a blood sample (3–6 ml) was drawn from the caudal vein by a syringe moistened with heparine. Eventual water in the intestine was drained and the fish weighed.

Analyses on gastric effluent. 2-ml aliquots of the effluent fluid from the stomach was titrated (Autotitrator assembly, Radiometer, Copenhagen) with 0.05 M sodium hydroxide. End point of the titration was the pH of the aquarium water (samples obtained before perfusion of the stomach with PR) or the pH of the solution used for perfusion of the stomach (during perfusion with PR). The acid content of each fraction was calculated from acidity and sample volume. The concentration of PR in each fraction was determined by reading the optical density (463 nm, isosbestic point, cf. Öbrink 1948) of 200 µl aliquots diluted with 2.0 ml 0.1 M HCl. An identically diluted aliquot of the perfusion fluid served as standard. The recovery of PR was then calculated and considered to mirror the recovery of secreted acid. In experiments where PR was used, only secretory data corrected for this recovery are presented. In experiments without PR, uncorrected values for acid output are given. Acid output is expressed as µmol/kg h, the BW obtained before surgery was used to calculate this.

For each animal, also the mean hourly recoveries of PR and of volume of the gastric perfundate for the whole perfusion period were calculated.

Analyses on blood plasma. Following centrifugation of the blood, the plasma samples were stored ...

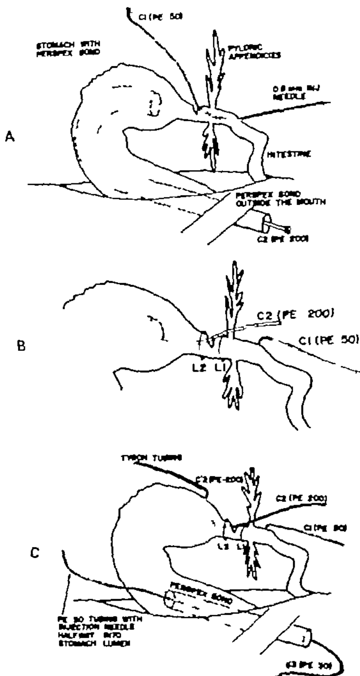


Fig. 1 A–C. Schematic illustration of the surgical procedure. Parts of catheters or bond drawn ... dashed ... the gastro-intestinal lumen.

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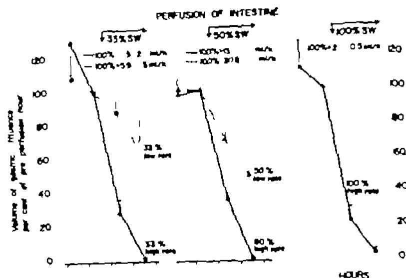
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Analyses on blood plasma Following centrifugatio f th blood, the plasma samples were stored ...



3 Effect of intestinal perfusion on volume of gastric effluence. Different rates of intestinal perfusion used, has calculated per kg of initial BW these were, in ml/kg h 18 5 ± 2.9 (33 high rate), 3 7 ± 0 6 low rate), 18 6 ± 2.3 (50 high rate), 2.9 ± 0.3 (50 low rate), and 13 6 ± 0.4 (100 high rate).

he perfusion of the intestine affected the effluent volume from the stomach. In experi-ts in which the intestinal perfusion started immediately following recovery from an-esia (and the perfusion of PR about 20 h later) most of the 20 first fractions were empty y occasionally a few drops were collected (not illustrated). In some other series the fusion of the intestine commenced at about 20 h postsurgically and PR 2 h later From 3 it will be seen that there was a drastic decrease in effluent volume from the stomach ring the first 2 h of intestinal perfusion. The effect is very conspicuous when a high rate 5 ml/h) of intestinal perfusion was used, less so when the perfusion fluid was delivered lower rate. It is to be noticed that the decrease in effluent volume occurs irrespective the degree of dilution of the perfusion SW The experiments depicted in Fig. 3 were then tinued for another 20 h, during which time PR was infused into the stomach. Then the hes were killed, BW estimated and bloodsamples drawn (Table I). For these initial PR periments, no acid secretory data will be presented.

During the study the technique was changed, and C'2 (instead of C2) was used to drain e stomach. This did not change the time-volume relationship (Fig. 2, lower panel, compare rves no injection C2' and 'no injection C'2'), but was technically easier and decreased e number of experiments which had to be discarded due to malfunction of the gastric nage. Neither did the use of C'2 change the effect of intestinal perfusion on the rate of stric volume effluence (Fig. 2). One series of fishes were intestinally perfused with 33 % W (high rate) between 22 and 24 h postsurgically During that time the effluent volume creased to zero in five of the eight fishes and to 0 18 ± 0.01 ml/h in the remaining three. 1 Fig. 2 (NaCl-injection C'2) is also shown that the i.m. injection of hypotonic saline (3.0 l/h, 0 6% NaCl) depresses the rate of effluent volume output.

The values for the recovery of gastric perfusion fluid (Table I) provide additional informa-

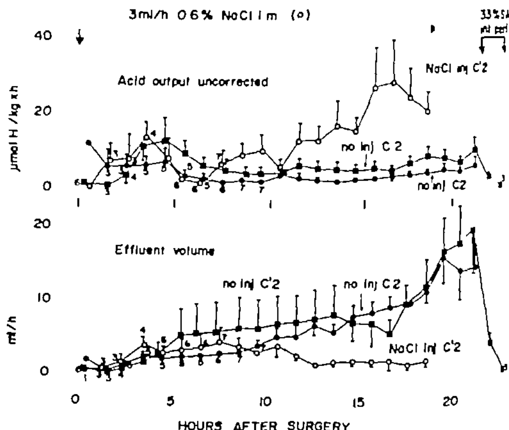


Fig. 2. Compariso of gastric acid secretion and gastric effluent volume for differently placed cath to drain the stomach (C2 and C'2). In one series, the fishes were i testinally perfused ith 33 ml/h, during 2 h. The effect of i.m. saline is also shown. Each group comprised 8 animals; the fishes delivering any gastric effluence has been indicated when less than 8

until assayed for osmolarity (freezing point depression, Knauer osmometer), Na and K -concentra (Turner Model 510 Flame Photometer) and Cl⁻-concentration (Radiometer CMT 10 Chloride Ti

Drugs Atropine sulfat hexamethonium bromide, sodium salt of phenol red (Sigma). Metiamide (Sm Kli e & French Laboratories). Heparine 5000 IE/ml (V trum). MS-222 (Sandoz).

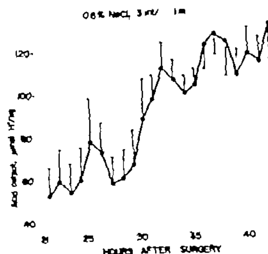
Statistics Values in the Figures nd the Table re mean and S.E. Group means were compared by t (Texas Instruments SR 52 calculator program ST1-07 two sample test, or ST1-06, paired observatio and linear regression calculated by the method of least squares (p ogram ST1-08).

Results

1 *Effluent volume*

In previous reports (Holstein 1975 1976, 1977) the term *drinking rate* was used to des the hourly volume output from the stomach. Since that volume, at least theoretical represents the sum of the positive terms *ingested water* and *volume secretion* from the st ach and a negative term representing *regurgitation* from the stomach, the term *effl volume* although certainly in essence mirroring the ingested volume, will be preferred.

When starting this study the catheter used to drain the stomach, though slightly d ferently placed, was of the same type as used previously and the results confirm ear observations. In experiments without perfusion of intestine or stomach, no gastric efflue or samples of very small volume were obtained for the first few h following recovery fr anaesthesia. Then the volume increased and finally stabilized at a level which varied fr individual to individual (Fig. 2, lower panel, no injection C2')



4. Gastric acid secretion in fishes ... injection of hypotonic saline ... experiment is ... continuation of one ... series in Fig. 2 (NaCl inj. C'2). ...

on the rate of oral water ingestion. A value in excess of 100% indicates that fluid has ... en added to the perfusion solution. As losses of fluid through the oesophagus could mask ... it, the figures for mean recovery of perfusion volume have to be corrected for recovery ... f PR. Of course, even the corrected figures must be carefully interpreted as gastric volume ... cretion also is included in the effluent volume. It will be seen that for most groups, about ... 5 ... of the perfused volume (8.5 ml/h) was recovered, indicating that drinking was low ... absent. A notable exception is the group perfused with 50% SW at a low rate, for which ... e recovered volume was 326%. The second highest value, 130%, belongs to the i.m. saline ... jected group.

Gastric acid secretion

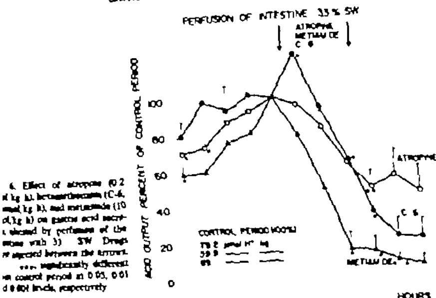
1. *Secretion before perfusion of the stomach with PR* (uncorrected values). In concert with ... revious findings, the secretion of gastric acid in pylorus ligated, non-perfused, non-injected ... shes was very low, not exceeding 10 μmol H ... /kg h. Two series of experiments are shown ... Fig. 2), in which the stomachs were drained by C2 and C'2, respectively. In one series ... C2), the fishes were killed at 22 h, their mean BW was then 90.5% of the initial value. In ... e other series (C'2), the experiment was terminated at 24 h, following two hrs of intestinal ... erfusion with 33 ... SW. This shortlasting perfusion had no effect on the BW (90.9% of ... initial ... alue), neither could any stimulation of the acid secretion be detected.

Fig. 2 also depicts the secretion of gastric acid in eight fishes i.m. injected with hypotonic ... saline from the start of the experiment. During saline injection the effluent volume was ... depressed, introducing a greater risk to underestimate the output of gastric acid (Holstein ... 1977). Nevertheless, an increased acid secretion during saline injection is obvious. The ... injection rate was chosen to deliver a volume sufficient to compensate for the decrease in ... BW in ... 450 g fish with ligated pylorus. This rate (3 ml/h) was, however, used for all fishes ... irrespective of weight and was found to counteract the weight loss encountered without ... saline injection. When killed (23 h later, the experiment was continued with PR perfusion of ... the stomach), the mean BW was 103.1% of the initial value.

Table 1 Final body weight, recoveries of phenol red and gastric perfusion volume, and plasma parameters.

Fig.	Treatment	n	% BW	Recoveries % PR	~ Vol	% vol 100 / % PR	Plasma parameters mOsm/l	Na (mM)	K (mM)	Cl⁻ (mM)
2	no treatment	8	90.5±0.9	—	—	—	384.5±4.2	201.5±3.8	5.3±0.5	166.3±2.5
2	Int. perf 33% last 2 h	8	90.9±0 4	—	—	—	364 6±4 1	199.2±4.7	3 6±0.1	172.8±2.3
2 & 4	0.6% NaCl i.m	8	103 1±0.7	92.8±2.1	120 8±8.1	130.2	327 1±2.9	190 0±2.0	2.3±0.1	152.4±1.3
3	Int. perf 100% (high rate)	5	86.1±1 8	72.8±7 3	83.2±7.0	114.3	530.3±5 4	242.3±12.7	5 6±0.2	209.5±11 0
3	Int perf 50% (high rate)	5	92 9±1 1	94.3±1 6	103 4±1 7	109 6	409 6±8 7	192.8±4.6	3 7±0.2	175 0±4 6
3	Int. perf 90% (low rate)	8	94 5±0.7	99.3±2.3	324 6±83 8	326.9	396.7±9.2	188.1±3.1	3.3±0.1	160 3±2.3
3	Int perf 33% (high rate)	5	101.2±0.3	90.2±5.0	98 9±6.2	109 6	342.9±9 8	153 6±12.5	3.2±0.3	162.8±7.3
3	Int. perf 33% (low rate)	5	97 9±1 0	—	100.4±10.0	—	355.0±13.2	172.2±7 6	3.4±0.1	157 6±6.1
5	Int perf 100	6	82.6±1 1	81 7±4.6	95.3±14.1	116.7	521.3±14.3	254 8±9 1	7 7±0.5	217 0±4 0
5	Int. perf 67%	4	89 6±1.3	83 4±1 3	90 4±6.8	107.2	446.5±17.2	191 8±6.6	4.2±0.5	221.3±12.6
5	Int. perf 50%	8	100 8±0.8	86 8±2.1	96.5±3 4	110.6	334.5±4.3	183.5±4.3	3.8±0.2	153 6±1.0
5	Int. perf 33%	10	103.3±0.9	94.9±1 9	113 0±8 8	119 1	375.9±3.3	179 9±4 8	3.3±0.3	156.9±1 4
6	Int. perf 33% atropine	10	103.3±0.6	88 7±2.6	96.6±2.9	108.8	360.4±6.7	184.7±6.8	3.0±0.1	168.6±5 1
6	Int. perf 33 hexamethonium	5	104 0±1.2	86.0±3.2	90.2±3.1	104.8	362.9±5 6	177 0±2.4	2.9±0.1	159 7±6.1
6	Int. perf 33% metiamide	6	102.0±1 0	89 9±6 0	96.2±5 6	107.0	340 1±9 1	173 6±9 0	3.3±0.2	152.0±4 6

I the experiments depicted Fig. 3 perfusion of the intestine started on the day afte surgery nd PR 2 h lat r I expts. shown i Fig. 5–6 in estl al perfusion started when the fishes had recovered from anaesthesia, d perfusion of the tomach with PR on the following d y



6. Effect of atropine (0.2 ol/kg h), hexamethonium (C-6, mol/kg h), and metiamide (10 ol/kg h) on gastric acid secretion elicited by perfusion of the intestine with 33 % SW. Drugs re injected between the arrows. ... significantly different om control period at 0.05, 0.01 d 0.001 levels, respectively.

V. Final BW, mean recoveries of gastric perfusion volume and PR, and plasma data appear Table 1.

In a previous report (Holstein 1977), the effect of atropine on histamine-induced gastric acid secretion was described. Atropine, 2 or 5 µmol/kg h was found to markedly depress the volume of gastric effluence, and the uncorrected output of gastric acid decreased. However when using PR (then injected at a rate of 0.62 ml/h) to correct for recovery no inhibition could be demonstrated. In the present work a higher rate of PR perfusion was employed (8.5 ml/h) and the dose-rate of atropine was lowered (0.2 µmol/kg h). Despite these precautions the hourly effluent volume in three of the eight fishes fell during atropine administration and the recovery of PR decreased to minimally 52, 31.6 and 70 % in the three fishes respectively. However following correction for recoveries the inhibition by atropine was still evident. Neither hexamethonium, nor methamide in the doses employed, caused any apparent changes in the effluent volume output.

In Fig. 7 the group means for plasma osmolarity, sodium-, chloride and potassium concentrations have been plotted against the corresponding group means for % BW. In all cases significant correlations were found. Values from three additional experiments in which the intestines were perfused with acidified SW or glucose (to be published separately) are included in Fig. 7.

Discussion

The present work has bearings on two problems, namely the regulation of gastric acid secretion and the regulation of oral intake of water in a marine teleost. The results strongly suggest that both are regulated by changes in the water balance.

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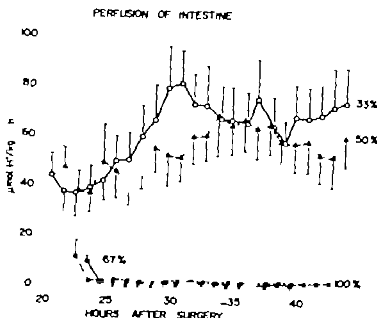


Fig. 5. Gastric acid secretion during perfusion of the intestine with pure sea water (100 SW) and variously diluted SW. For number of animals, see Table I.

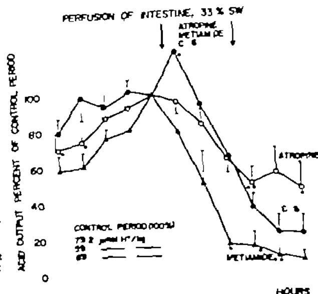
2.2. *Secretion during perfusion of the stomach with PR* (corrected values).

2.2.1 *Injection of hypotonic saline* The fishes used for this study had the C2 and C catheters placed only 3 ml/h of 0 6% NaCl was i.m injected from the start, and PR perfusion of the stomach commenced at 20 h. Gastric acid secretion during PR is shown in Fig. 4 postexperimental BW (103 1%) recoveries and blood data appear in Table I.

2.2.2. *Perfusion of the intestine* The results to be reported in this section concern fishes equipped with catheters C1 C2 and C3 The intestinal perfusion (high rate) started immediately following recovery from anesthesia, and perfusion of the stomach with PR about 22 h postsurgically In different experiments, the intestine was perfused with 100 ,, 67 50%, or 33% SW Fig 5 shows that the secretion was extremely depressed in fishes perfused with 100% or 67% SW and Table I shows that these groups were unable to maintain their BW at 100% Intestinal perfusion with 50% or 33% SW on the other hand, resulted in maintained BW and high rates of gastric secretion The first fractions following the start of PR perfusion of the stomach contained more acid than the following this is probably due to wash-out of acid accumulated during previous hrs.

When examinating the fishes postexperimentally it was noticed that the intestines of fishes perfused with 33% or 50% SW were heavily loaded with water while the 67%- and 100% SW-perfused fishes had remarkably empty intestines. It should also be mentioned that additionally two fishes were prepared for the 100% SW experiment, but these fishes died following 32 and 36 h of perfusion and were discarded.

2.2.3 *Effects of antagonists during intestinal perfusion* The fishes prepared for these experiments had catheters C1 C2, and C3 positioned The i m injection of the drugs which started at different h postsurgically lasted for three hrs. Gastric acid secretion, corrected for PR recovery is presented as the percentage of the h preceeding drug injection (Fig. 6) The absolute values for the respective control hour are given in the figure. Atropine (0.2 μmol/kg h) hexamethonium (C-6, 10 μmol/kg h), and metiamide (10 μmol/kg h) all significantly inhibited the gastric acid secretion during perfusion of the intestine with 33



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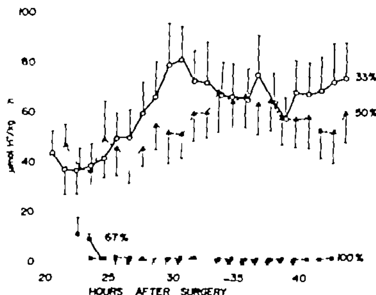


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plasma (Skadhauge 1969). In the present work, the steady state concentration of the intestinal content during perfusion was not determined, and it is only possible to conclude t the turning point, during the present conditions, corresponds to a value below 67% V probably between 50% and 33% SW

The commencement of intestinal perfusion resulted in a rapidly decreasing rate of effluent ume output from the stomach. In some experiments (e.g. Fig. 3) there elapsed about 20 h tween the observation on drinking rate and the sacrifice of the fish. It therefore remains ome uncertainty about the immediate effect of intestinal perfusion on the BW. However one series, the experiments were terminated following 2 h of intestinal perfusion with 33% SW. The final BW of this group (90.9 ±0.4%) did not differ from that of the unper- ued group (90.5 ±0.9%). Thus, the institution of 33% SW perfusion in previously un- fishes did not affect the BW within 2 h, i.e. the time period during which the effect drinking' was observed. There was, however, a significant difference ($P < 0.01$) in the sma osmolarity between the untreated group (384.5 ±4.2 mOsm/l) and the group in- tinally perfused with 33% SW during the last 2 h (364.6 ±4.1). Also plasma potassium is different between these groups ($P < 0.01$). That the decrease in any of these parameters ggers the interruption of oral water intake seems highly improbable, since the corre- onding parameters for the group i.m. treated with hypotonic saline—which maintained low rate of gastric volume output—exhibited still lower values. As 33% SW failed to fect the BW within 2 h, it seems highly unlikely that intestinal perfusion with more con- ntrated SW caused any initial, short-lasting increase in BW. Thus, in the case of 33% r 50% SW perfusion, drinking' was interrupted or decreased before any significant gain f water had been achieved, while perfusion with 67% or 100% SW precipitated drinking without ever alleviating the 'thirst'

The effect of intestinal perfusion on the rate of gastric volume effluence was independent f the degree of dilution of the perfusing SW but depended on the rate of perfusion. This ggests the participation of intestinal volume-sensitive receptors, the activation of which depresses the intake of water despite the continuous drive from a dipsogenic factor (vide infra). The conspicuous distension of the intestine following perfusion with 33% or 50% SW corroborates this interpretation, the equally conspicuous lack of fluid in the intestine following perfusion with 100% or 67% SW contradicts it. It must also be borne in mind that the effect on gastric effluent volume might be artifactual, elicited by mechanical de- formation of the stomach due to distension of the intestine. This assumption is strengthened by the great amount of water found in intestines of 33% and 50% SW-perfused fishes but contradicted by the lack of water in intestines of fishes perfused with more concentrated SWs. The persistency of the effect, despite change of technique to drain the stomach (C2 to C7) also does not favour the artifact theory

Referring to the preceding discussion, it seems unlikely that the depression of gastric effluent volume during i.m. injection of saline results from activation of the same mechanism which is responsible for the interruption of drinking when commencing intestinal perfusion. Speculatively the effect of i.m. saline is mediated by some change in plasma volume or composition, causing the withdrawal from the circulation of some factor stimulating water ingestion.

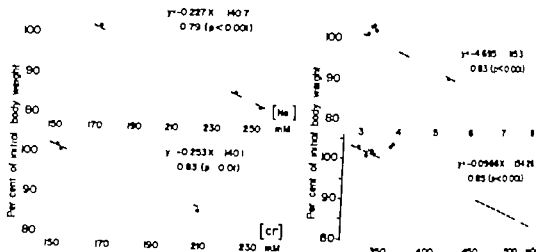


Fig. 7 Plots of different plasma parameters against final body weight expressed as a percentage of initial BW The graphs includes data from three experiments not reported in this communication symbols).

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Also *in vivo* experiments in the eel confirm the present observations. Skadhauge (19 perfused the intestine with 50% SW using a closed system where the water recirculate Before any net resorbtion of water took place, there was an initial dilution of the perfusi fluid. The cited works indicate that sea water following entry of the teleostean intestine, diluted with endogenous water before any net resorbtion of water is possible. When th flow reverses (turning point) the osmolality of the luminal content is higher than that

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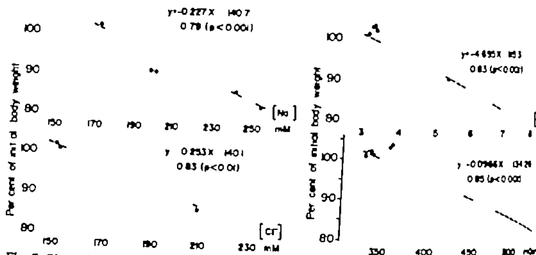


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physiological mechanism of value for an animal living in, and forced to drink of a hyp tonic medium.

If the high rate of acid secretion in the fishes which maintained their BW can be explai by the absence of an inhibitory factor the problem remains to find the agonist. Befo subjected to experimentation, the fishes were starved for at least one week. Therefore hypoglycemic secretion is possible. Experiments on vagotomized animals should help t clarify this point, and radioimmunologic methods should be employed to study the possi involvement of a peptide hormone. The experiments with antagonists (Fig. 6) offer r sufficient information to determine the nature of the secretory stimulus. As metiamide b not atropine inhibits histamine induced acid secretion in the Codfish (Holstein 1976, 197 it is only possible to exclude this amine from the list of possible agonists. The inhibito effect of atropine and hexamethonium suggests the participation of a nervous compone although the selectivity of hexamethonium as a ganglionic blocker must be interpreted w some care (Stenvenson and Grove 1977)

Finally the present results indicate that the basal acid secretion in the codfish is mu higher than was previously suggested (Holstein 1975) It is interesting that the pH (dete mined with a two-indicator paper) of the water film present on the gastric mucosa of starv Codfishes killed for other purposes was 2.1 ±0 7 n = 15 (Blychert and Holstein, unpublishe observations)

I am indebted to Mrs Inga Maj Örbom for e pert technical assistance to Mr Ingmar Hakenar for s plying fish, and t Smith, Kline & French Labs. for a gift of metiamide The work was supported by gr from Magnus Bergwalls Stiftelse and from Wilhelm och Martina Lundgrens V tenskapsfond.

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Synthesis of ³H acetylcholine in the rabbit lacrimal gland and its release by electrical field stimulation

By

JARL E. S WIKBERG

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Abstract

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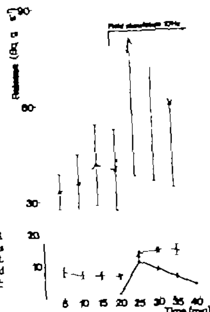
Electrical field stimulation was pplied to the rabbit lacrimal gland during incubation *in vitro* with choline, fter which the fflux of tritiated metabolites was studied In the presence of physostigmine acetylcholine was released into the incubation medium when the gland was stimulated electrically. T ide tity of the H-acetylcholine synthesized by the preparation was established by its identical ion exchange chromatographic properties to natl acetylcholine The tissue contents of ³H-acetylcholine and ³H-ch were 14 4 and 3 6%, respectively of th total activities found in the gland. This study demonstrates the rabbit lacrimal gland is innervated cholinergically It is suggested that the method developed might f value for studying effects of drugs n cholinergic neurotransmission in this organ.

The main secretory innervation of the mammalian lacrimal gland is thought to be cholinergic (Demtschenko 1872, Tepliachine 1894 Arenson and Wilson 1971) In previous studies the cholinergic neurotransmission in this organ measurement of tear secretion has generally been used for indirect determination of acetylcholine secretion from the nerves. Recently a radiochemical method for studying acetylcholine release from isolated guinea pig ileum was described (Wikberg 1977) In the present work a similar approach was used to study the release of acetylcholine from isolated rabbit lacrimal glands. Electrical field stimulation was applied to the organ during incubation with H-choline whereafter the release of H-acetylcholine synthesized by the organ could be examined.

Methods

Rabbits f either se were killed by blow on the neck nd xsanguinated. One of the lacrimal glands rapidly excised through the caudal supraorbital notch and ts capsule was carefully dissected away forceps. The orga (weighing 114 ± 11 mg), was mounted in a 4 ml water-jacketed perspex chamber containing Krebs solution (Wikberg 1977) with eserine sulphat 3 10^{-6} M The solution was kept at 37 C gassed with a mixture of O_2 (95%) nd CO_2 (5%)

The lacrimal gland was incubated with 9 3 10^6 Bq (becquerel, dps) of H-choline (methyl H-choline chloride 3 7 10^{12} Bq/mmole, Radiochemical Centre, Amersham, England) at a final concentration 1 10^{-6} M fo 1 h. During the incubation the organ was stimulated lectrically by 0.1 ms, 320 mA



1 Release of tritiated metabolites from the lacrimal d After incubation with H-choline the organ was ed for 45 min before any fractions were collected. se of acetylcholine (●—●), H-choline (○ ○) and F (▲—) are presented. The graphs show the mean ± of 5 expts.

polar pulses at 0 1 Hz by means of two 20 1 mm platinum electrodes mounted 10 mm apart on each of the organ, using Grass SD9 stimulator and home-constructed timer After the incubation the tric stimulation as stopped and the organ was washed with Krebs solution t intervals of 5 min for min, after hich 4 ml fractions ere collected for analyses.

iochemical assay

method described previously (Wikberg 1977), with some minor modifications, was used, to separate radioactivity of the samples into 3 fractions: ³H-acetylcholine, H-choline and fraction hich remained precipitated by ammonium reineckate (UPF) In order to improve the paper chromatographic separation acetylcholine and choline, descending rather than the ascending system with acetone/water/10 N 3 100 20 1 (by volume) as used. The chromatograms were allowed to run for 10 h, using the dorch- T technique T facilitate the identification of carrier choline and acetylcholine the spots were developed spraying ith an iodoplatinate reagent (Walsh *et al.* 1967). By this method the absolute distance ween the spots was 3 cm In order to elute the radioactivity from the paper and to decolorize the iodo- tinate reagent, mixture of 3 ml ethanol/0.5 M NH_3 1 1 (v/v) was used. These samples could be counted w addition of Instagel® (Packard Instrument Company Inc) as described previously

After the expt the lacrimal gland was frozen in Prigen solid CO_2 and kept at −80°C until analysed. e tissue was homogenized with glass homogenizer in 0 4 M $HClO_4$ (1+0 5 ml) at 0°C and then centri- ged at 10 000 g for 30 min. The pellet was washed once with 1 ml of the HClO solution. The pooled ernatant was diluted to 25 ml and 1 ml fractions were taken for analyses as described above for the thing solution The pellet as dissolved by adding 2 ml Soluene 350 (Packard Instrument Company) and counted in Packard T Carb 3375 liquid scintillation spectrometer after addition of toluene ed scintillator

Ion exchange chromatography

approach similar to that described previously was used (Wikberg 1977) Columns measuring 4 4 430 ere packed ith Amberlite CG 50 II (200–400 mesh) equilibrated with 0.1 M sodium phosphate ffer pH 7 0 The samples applied on the column ere eluted with 5 ml H_2O followed by the phosphate ffer at flow rate of 5 ml/h. I order to determinate the retention volume of acetylcholine, 1 3 10⁴ Bq ¹⁴C-acetylcholine (Acetyl-1 ¹⁴C-acetylcholine iodide 8 5 10¹⁰ Bq/mmole, NEN) was added to the sample d the radioactivity as separated from the tritium activity by measurement at separate channels in the

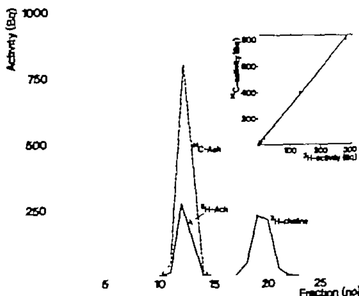


Fig. 2. Ion exchange chromatography of the reineckate-precipitated fraction in the washing solution l the lacrimal gland The medium was collected during electrical field stimulation at 10 Hz and the quater ammonium compounds were precipitated by addition of mmonium-reineckate in the presence of a acetylcholine and choline After batch treatment with Biorex 9 i water and addition of 1 3 H ¹⁴C-acetylchol ne as a marker the sample w s applied on the column, followed by elution ith 5 ml and 0.1 M phosphate buffer pH 7.0. Insert Correlation between the H-activity in the first peak and ¹⁴C-activity of the ¹⁴C-acetylcholine marker (= 1.000).

liquid scintillation spectrometer The data btained were corrected for quenching nd for overlap be channels, using a internal standard and a double isotope computer programme written for HP The counting efficiency was 15% for H and 53% for ¹⁴C.

Results

During unstimulated conditions almost no tritiated acetylcholine was released from organ After the 45-min washing period when collection of fractions was started, only of the radioactivity released to the bathing solution consisted of H-acetylcholine. Mo the activity at this time consisted of UPF (79%), followed by H-choline (19%). Elect field stimulation with 1 0 ms pulses at 10 Hz induced a 21-fold increase in the release of acetylcholine. The release of acetylcholine was not maintained at that level but decreas half its maximal value in approximately 10 min, despite continuous electrical stimula The electrical stimulation also induced a two-fold increase in the release of H-choline, during the subsequent stimulation periods this release showed a smaller tendency to de than that of H-acetylcholine The release of UPF also increased approximately 2-fol electrical stimulation (Fig. 1)

The identity of H-acetylcholine released during electrical stimulation was verified by exchange chromatography After application of the reineckate precipitated fraction o column two peaks of radioactivity appeared The first peak showed excellent correl to the marker ¹⁴C-acetylcholine peak (correlation coefficient 1 000) (Fig. 2)

[illegible] I. Tritiated metabolites present in the lacrimal gland at the end of the expt. The radioactivity was separated into 4 fractions: the ammonium reineckate unprecipitated fraction (UPF), acetylcholine (Ach), choline (Chol) and the perchloric acid insoluble fraction (Pellet). The table gives the mean ± S.E. of 5 expts.

	10⁻ Bq	
[illegible]	7.55 ± 1.02	38.1
[illegible]	2.85 ± 0.35	14.4
[illegible]	0.72 ± 0.07	3.6
[illegible]	8.68 ± 1.44	43.8

[W]hen the expt. started the tissue content of tritium was 2.18 ± 0.21 10⁴ Bq. This amount [cor]responded to 23.4% of the total activity of ³H-choline added to the incubation medium [at] the start of the expt. During the course of the expt. a total of 1.99 ± 0.60 10⁴ Bq of [triti]um was released from the lacrimal gland. The secretion of ³H-acetylcholine was 1.22 ± [...]0 10³ Bq, of ³H-choline 2.93 ± 0.30 10³ Bq and of UPF 1.64 ± 0.77 10⁴ Bq. At the [en]d of the expt. a considerable amount of ³H-acetylcholine was present in the tissue but the [³H]-choline content was low. The largest amounts of activity however were found as UPF [an]d in the PCA-insoluble fraction (Table I).

Discussion

[T]he innervation of the mammalian lacrimal gland has been studied by histological techniques [in] several earlier studies. Ichikawa and Nakajima (1962) showed that the lacrimal gland of [th]e rat contains unmyelinated nerve fibres in the inter- and intra-lobular connective tissues. [E]lectron microscopic studies on lacrimal glands of monkeys have indicated that para[sy]mpathetic nerve fibres originating in the sphenopalatine ganglion innervate interstitial [a]nd parenchymal tissues (Ruskell 1969). The arteriole of the monkey lacrimal gland may [re]ceive both cholinergic and sympathetic nerves (Ruskel 1967, 1969). Histochemical staining [fo]r cholinesterase in the human lacrimal gland has revealed choline esterase activity in [m]yoepithelial cells and nerve fibres surrounding the acinar cells (Mizukawa *et al.* 1962).

The present study demonstrates that the rabbit lacrimal gland is capable of accumulating [tri]tium when incubated with ³H-choline. It may be estimated that a passive uptake of [c]holine of about 5 of the amount in the incubation medium will take place under the [ex]perimental conditions used. The high uptake observed (23.4% of the given amount) there[fo]re suggests that an active mechanism for choline transport into the tissue is present. This [tr]ansport system could be located in nervous tissue as well as in glandular cells. Active up[ta]ke of choline into nervous tissue is well documented (Pert and Snyder 1974, Simon *et al.* [1]976, Wikberg 1977), but other cells such as human red blood corpuscles may have transport [sy]stems for choline accumulation (Clement and Colhoun 1975).

In this work it was found that the rabbit lacrimal gland is capable of synthesizing ³H-[a]cetylcholine from ³H-choline. The chemical identity of ³H-acetylcholine seems to be [c]ertain, since the metabolite showed properties identical to those of native acetylcholine i[n] [tw]o different chromatographic systems. The basal release of ³H-acetylcholine into the in-

cubation medium was almost negligible This contrasted markedly with the results obtain from guinea pig ileum (Wikberg 1977) where the unstimulated release was much higher r showed spontaneous fluctuations Electrical stimulation with stimulation parameters indic ing that selective stimulation of nervous tissue was induced, resulted in a large increase the release of H-acetylcholine from the lacrimal gland The results of these experime may be taken as fairly clear evidence of the presence of cholinergic innervation in this tiss

The tissue content of H-acetylcholine was more than 20-fold larger than the amount H-acetylcholine released during the electrical stimulation In spite of this the release of acetylcholine declined rapidly after the initial large release This observation might indica that different compartments of acetylcholine are stored in the nerves, with variable capaci to release upon stimulation Collier and MacIntosh (1969) have suggested that the acety choline turn-over in the superior cervical ganglion in cats takes place in non-uniform po and that the newly synthesized acetylcholine shows a preferential release upon preganglio stimulation An alternative explanation for the decline of the release observed in this stu may be that a regulatory mechanism is present which after a time diminishes the release acetylcholine.

Besides H-acetylcholine, H-choline was also released into the incubation medium. As rather long washing period (45 min) had been used before any fractions were collected i probable that the H-choline was derived predominantly from intracellular sources. Th tissue content of H-choline at the end of the experiment was rather low however wh might indicate that H-choline was derived from some other kind of radioactive metabolit It seems improbable that the H-acetylcholine released was responsible for the H-chol formation, since a rather high concentration of acetylcholine-esterase inhibitor was used a the release of the two metabolites showed a poor temporal correlation (Fig. 1)

Other metabolites were also present in the washing medium and in the tissue. This stu gives no definite information on the nature of UPF It might represent such metabolites phosphorylcholine, betaine or N N-dimethylethanolamine. It has been suggested that phosphorylcholine and H-betaine may be synthesized from H-choline in a variety of tiss (Sung and Johnstone 1965 Collier and Lang 1969 Szerb 1975).

In conclusion, it is proposed that the use of H-choline for labelling of intrinsic acety choline stores with tritium might be of value for studying acetylcholine release from the gland Recently this approach was used to investigate the influence of β-adrenoceptor act drugs on the cholinergic transmission in the rabbit lacrimal gland (Åberg *et al* 1978).

I am indebted to Assistant Professor Rolf G G Andersson for valuable criticism of this work. Finan support was given by the Swedish Medical Research Council (B78-04X-04498-04).

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A healing promoting factor in rat wound fluid

By

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The origin and nature of stimulus to cells and tissues to repair after an injury is incomple[illegible] known The stimulation for wound-healing may be due to a positive, healing-promot[illegible] factor Such substances may be formed and released either systemically or locally The [illegible] of the present study was to examine whether a healing-promoting factor is formed or [illegible] and, in the former case, if it increases the tensile strength. This may be used as a measur[illegible] indicator of the extent of the repair of the tissue injured (Howes, Sooy and Harvey 19[illegible] Sandblom, Petersen and Muren 1953).

Fluid produced in a stainless wire mesh cylinder subcutaneously implanted, is [illegible] interstitial wound fluid (Schilling, Joel and Shurley 1959 Hunt *et al* 1967). If a stimula[illegible] factor is liberated locally it may exist in this fluid. The tensile strength of wounds to wh[illegible] such fluid was added, was determined. An increase would indicate the presence of a heali[illegible] promoting factor

The formation of granulation tissue of the healing wound is considered to increase if a stimulating factor exists. Therefore, cellulose sponges were implanted in wounds to [illegible] this effect assuming that increases in weight are due to stimulation of connective tis[illegible] formation (Grindlay and Wangh 1951)

Rabbits weighing about 3 kg were used. The animals were anesthesized with Nebunal® (30 mg/kg b[illegible] Two incisions about 2 cm lo g were made o each side of the middle line of the back. Through each inc[illegible] a stainless wire mesh cylinder (5 cm long and 2 cm in diameter) was mplanted s.c. The incision [illegible] by single silk suture (3/0). One week later the cylinder was punctured and the fluid collected and [illegible] wound fluid. Serum was received by puncture of ear veins of rabbits.

3 longitudinal skin ncisions of a length of 4 cm and reaching underlying muscular fascia were made each side of the back of rabbits at distance of 2.5 cm from the middle line. 2 ml of serum [illegible] t the wounds on one side, and 2 ml of the wound fluid to the contralateral wounds. The [illegible] closed by 3/0 silk.

The tensil strength of the wounds were determined by the method described by Sandblom *et al.* ([illegible] one week after the incision. The rabbit was anesthesized and the sutures removed prior to the [illegible] ment. The wound was ruptured on either sid of t. Two determinations were made of the tensile stre[illegible] of each wound.

Viscose cellulose sponges 10 10 20 mm (Visella Säteri Oy Valkeakoski, Finland) were divided [illegible] two equal parts and immediately sew together w th silk suture. The pair implanted in each animal [illegible] of about the same weight. The animals were anesthesized with the and two symmetrical incisions [illegible] to the midline of the back were made A s.c. pock t was dissected i each wound. The cellulose spo[illegible] had been soaked in serum or wound fluid prior to the implantation in the pockets. Each animal got [illegible] treated sponge on one of the pock ts and wound fluid-treated sponge in the other

...e I

	T.S.	Significance	N
	553 ± 25	p 0.01 0.02	68
...trol	466 ± 25		68
	454 ± 21	p > 0.20 0.30	78
...m	418 ± 19		78
...m	405 ± 23		71
...trol	420 ± 23		68

...ensile strength of wounds exposed to wound fluid (WF), serum or control expressed as g. Mean ± S.E. calculation of significance was made using Students' t-test. N = number of observations.

7 days later the sponges were removed, and their surfaces dissected free from surrounding tissue. The ...ture of the sponge was taken away and the tensile strength between the two halves was measured. The ...y weight of the sponge was determined after drying at 70° in vacuum desiccator over phosphorous ...itoxide for 3 days.

...e tensile strength of the wound on each side was compared to that of the wound on the ...ntralateral side. It was increased in wound exposed to wound fluid, compared to both ...rum and control, i.e. no treatment. The difference was most pronounced in the group ...ound fluid/control (Table I).

The dry weight of the sponges did not show any differences in either groups. The same ...as true for the tensile strength of the sponges implanted under the conditions tested.

Many factors effect wound-healing in animals, such as the general health, the nutritional ...atus and lack of certain vitamins. Each animal has its own characteristic response to an ...jury. Considering these facts, the present experiments were performed on animals which ...rved as their own control. The exact mechanism could not be demonstrated in the present ...udy. It was, however, possible to prove that wound fluid *per se* promotes the reparative ...rocess.

The reason for the increased tensile strength of skin wounds exposed to wound fluid apparently is a change in the connective tissue formed (Dunphy 1960). Its nature is not known. Maybe there is a more extensive proliferation of connective tissue cells and formation of ...ollagen (Sandberg and Lederfeldt 1963). However, the dry weight of the sponges did not ...ncrease significantly, probably reflecting that the testing method was not sensitive enough. The tensile strength of the sponges, furthermore, did not show any difference after treat...ment with wound fluid or serum compared to no pre-treatment, although the pores in the ...ponges were filled with cells, fibres and amorphous material.

The type of cell proliferating in repairing wounds is of importance. It is likely that they ...roduce or induce production of a factor increasing the tensile strength. The nature of such ...a factor is not known. It is likely to be formed locally in the wounded area. Most cells are ...ormed in repairing tissue. It is tempting to assume that histamine and biogenic amines may ...be of importance in healing wounds (Kahlson and Lederfeldt 1960, Sandberg 1962, Sandberg and Lederfeldt 1963, Wasilewski *et al.* 1965).

Growth-promoting factors have been demonstrated in several different systems. Pr hepatectomi results in liberation of a factor which is humoral It may increase the freque of mitosis even in a normal animal transfused with blood from another one subjected partial hepatectomi (Christensen and Jacobsen 1950 Bucher Scott and Arb 1951 Wenneck and Sussman 1951) If embryonic tissue is transplanted to adult animals, this starts to d ferentiate in the new host. Another such factor is the nerve growth factor which indu a proliferation of sympathetic neurons but not of motor neurons. This latter factor is kno to be a polypeptide (Levi Montalcini 1973). Thus the conclusion that wound fluid r contain a healing promoting factor of unknown composition, influencing the repai process in connective tissue

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The predominanting molecular form of gastrin and cholecystokinin in the gut is a small peptide corresponding to their COOH-terminal tetrapeptide amide

By

JENS F. REHFELD and LARS-INGE LARSSON

ptide hormones in tissue and blood are analyzed by immunochemical methods. In view the molecular heterogeneity of each hormone, the structural homology between groups hormones, and the occurrence of hormonal peptides in nerves, immunochemical measurents require exact knowledge of the sequence for which an antiserum is specific. We have cently developed a panel of sequence-specific antisera for radioimmunoassay (Rehfeld 78 a) and immunocytochemistry (Larsson and Rehfeld 1977) of the two related peptide rmones, gastrin and cholecystokinin (CCK). Using this panel to monitor fractionations, noted that antisera specific for the common COOH-terminal tetrapeptide amide sequence tected a new small component in extracts from the gut (Rehfeld 1978 b). Moreover the OOH-terminal-specific antisera demonstrated more cells than were seen with antisera ainst NH_2-terminal and middle regions of $gastrin_{17}$, CCK_8 and CCK_{33} (Larsson and ehfeld 1979). The COOH-terminal-reactive cells that contained neither CCK_{33}, CCK_8 nor $astrin_{17}$ represents a new type of gastrointestinal endocrine cell (the TG-cell), which has w been identified t the electron microscopical level (Larsson and Rehfeld 1979 and Fig. From antral extracts we purified the new component and demonstrated its identity with tetrapeptide amide common to gastrin and CCK (unpublished). In the following, we port the distribution and concentrations of the tetrapeptide amide in the gastrointestinal act

Mucosa from the gastrointestinal tract of three pigs was collected at a local abbatoir 20 n post mortem. The mucosa was immediately frozen on dry ice and stored at −80°C. hile frozen the mucosa was cut into small pieces and extracted in boiling water and acetic ad, as described in detail elsewhere (Rehfeld 1978 b). 2–6 ml extract was applied to high-solution Sephadex columns (G-50 superfine, 25 2 000 mm), eluted and calibrated as scribed in detail elsewhere (Rehfeld 1978 b). The immunoreactivity in extracts and chromagraphic fractions was quantitated by an assay specific for the COOH-terminal sequence f gastrin and CCK using antiserum 2609 (Rehfeld *et al* 1972, Rehfeld 1978 a) monodinated $gastrin_{17}$ and highly purified porcine $gastrin_{17}$, CCK_{33}, and synthetic tetrapeptide nide as standards.

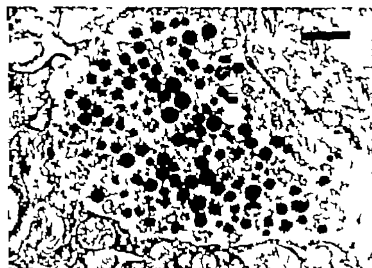


Fig. 1 Electron micrograph of immunocytochemical ... TG cell process in the duod... Brunner gland area of a ... monkey Bar indicates 1 μm

Table I shows that the tetrapeptide-like component on a molar base predominates throughout the gastrointestinal tract in comparison with gastrin , and CCK_{33}. In antrum gastrin was present in almost half the concentration of that of the tetrapeptide-like component whereas the tetrapeptide predominated even more in distal regions of the gut.

Since the tetrapeptide amide contains essentially all the biological information present in gastrin and CCK and since the tetrapeptide-like component predominates in all regions of the gut, it is tempting to suggest that the new fifth component of gastrin and CCK is the principal molecular form of both hormones. It is possible that the tetrapeptide amide an

TABLE I Regional concentration (pmol/g mucosa (wet weight)) of gastrin , cholecystokinin and the common COOH terminal tetrapeptide amide in the porcine gastrointestinal tract (mean ± S (n = 3).

	G	G/CCK_4	CCK_{33}
Antrum	5 921 ± 312	13 170 ± 5 070	< 0..
Duodenum (proximal)	3 1 ± 1.0	8 220 ± 3 180	509 ± 183
Duodenum (middle)	1.2 ± 0.5	10 740 ± 3 410	650 ± 115
Duodenum (distal)	< 0.2	3 810 ± 960	82 ± 19
Jejunum (proximal)	< 0.2	2 790 ± 1 040	478 ± 176
Jejunum (middle)	< 0.2	1 180 ± 470	327 ± 87
Jejunum (distal)	< 0..	108 ± 51	16.1 ± 6.0
Ileum (proximal)	< 0.2	291 ± 92	< 0.2
Ileum (middle)	< 0.2	113 ± 17	< 0.2
Ileum (distal)	< 0.2	48 ± 22	< 0.2
Colon (proximal)	< 0.2	59 ± 18	< 0.2

rts its effects locally by paracrine secretion, since it may be cleared from circulation in the r In the colon of most species gastrins and CCKs are localized exclusively to neurons nis-Wallensten *et al* 1977 Larsson and Rehfeld 1978 b). Thus the tetrapeptide is of neuronal origin, as previously demonstrated in the central nervous system (Rehfeld 8 b, Larsson and Rehfeld 1978 b). Due to its high concentration and small size the apeptide may well turn out to be the principal neurotransmitter of the gastrin-CCK ily

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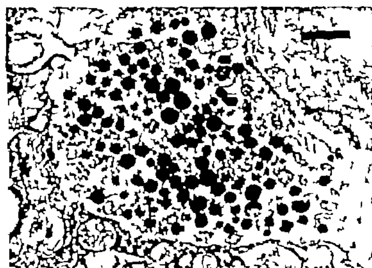


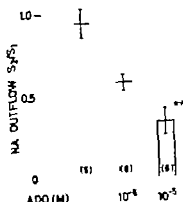
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Ileum (middle)	< 0.2	113 ± 17	< 0.2
Ileum (distal)	< 0.2	48 ± 22	< 0.2
Colon (proximal)	< 0.2	59 ± 18	< 0.2



1. Effect of adenosine (ADO) on NA release in response ...sympathetic nerve stimulation (10 Hz, 30 s) in perfused ...t hearts. Values presented as ratio between two consecu... stimulations and given as means ± S.E. Figures ...ithin ...ntheses: number of expts.

...range adenosine markedly and dose-dependently depressed the release of NA elicited ... adrenergic nerve stimulation (Fig. 1). The mechanical responses were also depressed. ... adenosine effects were rapidly reversed when the infusion was discontinued. Adenosine ...μM inhibited the chronotropic and inotropic responses to nerve stimulation 19.7 ± 3.4 ... 39.2 ± 4.0 (means ± S.E., n = 7) percent, respectively. At this concentration adenosine ...se had no effect on the contractile force and only inconsistently and slightly (less than ...) depressed the heart rate. Seemingly adenosine 10 μM more effectively depressed the ...chanical responses to nerve stimulation. However quantitative analysis of these data ...d not appear meaningful since 10 μM adenosine *per se* markedly depressed heart rate and ...ntractile force.

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Inhibitory effect of adenosine on adrenergic neuroeffector transmission in the rabbit heart

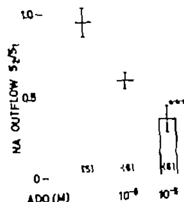
By

PER HEDQVIST and BERTIL B. FREDHOLM

Adenosine is a potent coronary vasodilator and has been implicated as a mediator of cardi blood flow regulation (Wedd 1931 Berne 1963 Rubio and Berne 1969). Furthermor adenosine decreases heart rate (Wedd 1931) and antagonizes chronotropic and inotro responses to catecholamines (Schrader *et al* 1977). Recent observations indicate t adenosine may inhibit noradrenaline (NA) release in a number of tissues, such as kidn adipose tissue, vas deferens and vascular strips (Hedqvist and Fredholm 1976, Enero a Saidman 1977 Verhaege *et al* 1977) The present study indicates that such a mechanism operative also in the heart.

Rabbits of either sex weighing 1.5–2.0 kg, received heparin 1 000 IU/kg b.wt. i.v. a were killed by a blow on the head After exsanguination the heart with its sympathe nervous supply was dissected free, as described by Huković and Muscholl (1962) and w perfused according to the Langendorff technique with Tyrode's solution, at a const pressure of 75 cm H_2O of the following composition (conc. in mM) NaCl 136.7 KCl 2 $CaCl_2$ 1 8 $MgCl_2$ 1 0 $NaHCO_3$ 11 9 NaH_2PO_4 0 4 glucose 5 5 ascorbic acid 0.1 and k at 37°C and gassed with 5% CO_2 in O_2. The sympathetic nerves were pulled through platin ring electrodes and were stimulated with two Grass S4 stimulators, delivering 30 s trains biphasic pulses (10 Hz, 1 ms, 20 V effective strength) at intervals of 10 min The myocardi contractile force and heart rate were monitored by means of a force displacement transdu (Grass FT 03) connected to a hook in the ventricle, or a pressure transducer (Statham P Dc) connected to a small rubber balloon in the left ventricle. Either parameter was displ on a Grass polygraph. Perfusate from the heart was collected for two min immedia preceding a stimulation, and during and 90 s after the stimulation The NA content in perfusate samples was determined fluorimetrically according to Euler and Lishajko (19 Adenosine was infused into the perfusion stream close to the heart to give a final concent tion of 1 or 10 μM

In the Langendorff-perfused rabbit heart adenosine (1 and 10 μM) *significantly* incre coronary drainage in agreement with previous reports that adenosine is vasodilator in mammalian heart (Wedd 1931 Berne 1963). The two concentrations caused 29 1 ± 6.1 a 33.2 ± 4 1 (means ± S E., n = 8) percent increase in flow rate, respectively indicating t maximal vasodilatation was achieved already with 1 μM adenosine. In the same concen



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From the Department of Physiology Karolinska Institutet, Stockholm, Sweden

Inhibition of thirst and apparent ADH release by intracerebroventricular ethacrynic acid

By

M RUNDGREN, M J MCKINLEY L. G LEKSELL and B. ANDERSSON

lies performed in the goat over the past several years imply that cerebral receptors ulating water intake and the release of antidiuretic hormone (ADH) have a juxtaventri- ar location, and that the activity of these receptors is positively correlated to the [Na] the cerebrospinal fluid (CSF) That Na -transporting enzyme activity in some way may essential for the excitation of the receptors is indicated by the observations that intra- ebroventricular (IVT) infusions of certain stimulators of active Na -transport interact h CSF Na in eliciting thirst and ADH-release (cf Andersson 1978). This possibility also supported by effects of Na–K-ATPase inhibitors so far reported in three species. the rat hypothalamic implants of ouabain suppressed water intake (Bergmann *et al* 1967) d induced a water diuresis (Gutman, Bergmann and Zerachia 1971). In the sheep the racarotid infusion of ouabain virtually abolished water intake in response to intracarotid usions of hypertonic NaCl and angiotensin II and decreased the urge to drink in water pleted animals (Weisinger Denton and McKinley 1977). In the goat, IVT infusions two weaker inhibitors of Na–K-ATPase (glycerol and deuterium) prevented drinking in ater-deprived animals and attenuated the basic ADH secretion of goats in normal water lance (Olsson, Larsson and Liljekvist 1976, Leksell, Lishajko and Rundgren 1976). erefore, it was of interest to study whether another inhibitor of enzymatic Na -transport, hacrynic acid (cf Schwartz, Lindenmayer and Allen 1975), would affect the cerebral ntrol of water balance in a similar manner

T o female, and two castrated male goats (b.wt. 31 36 kg) were used. The animals were upplied with permanent platinum-iridium cannulas (Åkerlund, Andersson and Olsson 1973) the lateral ventricle where IVT infusions were made at a rate of 20 µl/min. The sodium lt of ethacrynic acid (Edecrine, Merck S & D") was used for IVT infusions.

Thirst. In the castrated male goats IVT infusions of ethacrynic acid (0.3 µg/kg min⁻¹) ssolved in 0.3 M NaCl, or merely 0.3 M NaCl were alternatingly made for 60 min. During he first 55 min the water bucket in front of the goats was covered with a lid. This lid was mporarily removed at 10 min intervals to test the willingness of the goats to drink. They

Holder of an Australian National Health and Medical Research Council C. J Martin Fellowship. On ave from the Howard Florey Institute of Experimental Physiology and Medicine, University of Mel- bourne

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[Stud]ies performed in the goat over the past several years imply that cerebral receptors [regu]lating water intake and the release of antidiuretic hormone (ADH) have a juxtaventri[cula]r location, and that the activity of these receptors is positively correlated to the [Na] [in t]he cerebrospinal fluid (CSF). That Na -transporting enzyme activity in some way may [be e]ssential for the excitation of the receptors is indicated by the observations that intra[cere]broventricular (IVT) infusions of certain stimulators of active Na -transport interact [wit]h CSF Na in eliciting thirst and ADH-release (*cf* Andersson 1978). This possibility [is a]lso supported by effects of Na-K-ATPase inhibitors so far reported in three species. [In] the rat hypothalamic implants of ouabain suppressed water intake (Bergmann *et al.* 1967) [and] induced a water diuresis (Gutman, Bergmann and Zerachia 1971). In the sheep, the [intra]carotid infusion of ouabain virtually abolished water intake in response to intracarotid [infu]sions of hypertonic NaCl and angiotensin II and decreased the urge to drink in water [dep]leted animals (Weisinger Denton and McKinley 1977). In the goat, IVT infusions [of] two weaker inhibitors of Na-K-ATPase (glycerol and deuterium) prevented drinking in [wa]ter-deprived animals and attenuated the basic ADH secretion of goats in normal water [ba]lance (Olsson, Larsson and Liljekvist 1976, Leksell, Lishajko and Rundgren 1976). [Th]erefore, it was of interest to study whether another inhibitor of enzymatic Na -transport, [et]hacrynic acid (*cf* Schwartz, Lindenmayer and Allen 1975), would affect the cerebral [co]ntrol of water balance in a similar manner

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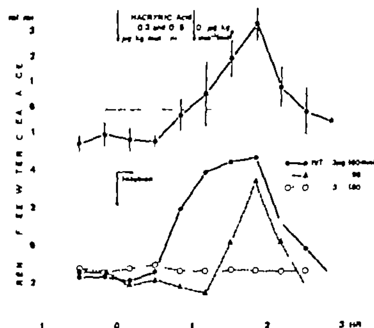


Fig. 1 *Above* Mean effect of free water clearance of etha- acid infusions into the lateral ventricle (n = 4) of two normohydrated goats. Vertical lines S.E. *Below* The separate responses to IVT ethacrynic acid 0.3 and 0.1 μg/kg min^{-1} in one of the animals. For comparison shown the absence of diuresis in response to an intravenous infusion of the larger dose

were not allowed to consume any water however After 55 min the goats got free access to water Both kinds of infusion induced water seeking behaviour within 10 min. However during the ethacrynic acid/NaCl infusions the urge to drink disappeared 20 to 40 min later Hence, no water was drunk when the animals got free access to it. In contrast, when 0 3 M NaCl was infused the goats drank considerable amounts of water at this stage (see table below) The ethacrynic acid infusions did not affect the appetite of the goats or cause any other visible signs of discomfort

Lateral ventricle (20 μl/min 60 min)	Water intake at 55 min (ml, Mean ± S E.)	Exp no.
0.3 M NaCl	860 ± 170	6
Ethacrynic acid in 0.3 M NaCl	0	6

ADH secretion In the two female goats ethacrynic acid was infused into the lateral ventricle dissolved in slightly hypertonic (0 17 M) NaCl In one of the goats the doses were 0.3 and 0.15 μg/kg min (duration 60 min) and in the other animal 0.3 and 0.1 μg/kg min (duration 60 and 90 min respectively). All infusions induced positive renal free water clearance (C_{H_2O}) towards the end of the infusion period. However at the lowest acid concentration C_{H_2O} did not become positive until after about 80 min of infusion Intravenous control infusions (0.3 μg/kg min for 60 min) did not induce any water The mean effect on renal C_{H_2O} and the individual results in one of the goats are shown Fig. 1 Neither the IVT nor the i v infusions had any obvious effect upon renal Na and K excretion.

Discussion This study shows that the administration of systemically inactive amounts ethacrynic acid into the CSF of the lateral ventricle can block the dipsogenic

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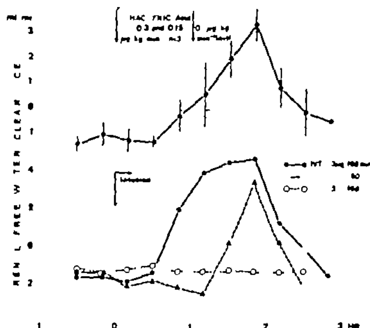


Fig. 1 *Above* Mean effect ... free water clearance of ethacrynic acid infusions into the lateral ventricle (=4) of two normally nonhydrated goats. Verticals S.E. *Below* The separate responses to IVT ethacrynic acid 0.3 and 0.1 μg/kg min^{-1} ... the animals. For comparison shown the absence of ... in response to an ... infusion of the larger dose

were not allowed to consume any water however. After 55 min the goats got free access to water. Both kinds of infusion induced water seeking behaviour within 10 min. However during the ethacrynic acid/NaCl infusions the urge to drink disappeared 20 to 40 min ... Hence, no water was drunk when the animals got free access to it. In contrast, when ... 0.3 M NaCl was infused the goats drank considerable amounts of water at this stage (table below). The ethacrynic acid infusions did not affect the appetite of the goats or ... any other visible signs of discomfort.

Lateral ventricle (20 μl/min 60 min)	Water intake at 55 min (ml Mean ± S.E.)	Exp no.
0.3 M NaCl	860 ± 170	6
Ethacrynic acid in 0.3 M NaCl	0	6

ADH secretion In the two female goats ethacrynic acid was infused into the lateral ventricle dissolved in slightly hypertonic (0.17 M) NaCl. In one of the goats the doses were 0.3 and 0.15 μg/kg min (duration 60 min) and in the other animal 0.3 and 0.1 μg/kg min (duration 60 and 90 min respectively). All infusions induced positive renal free water clearance (C_{H_2O}) towards the end of the infusion period. However at the lowest ethacrynic acid concentration C_{H_2O} did not become positive until after about 80 min of infusion. Intravenous control infusions (0.3 μg/kg min for 60 min) did not induce any water diuresis. The mean effect on renal C_{H_2O} and the individual results in one of the goats are shown in Fig. 1. Neither the IVT nor the i.v. infusions had any obvious effect upon renal Na and K excretion.

Discussion. This study shows that the administration of systemically inactive amounts of ethacrynic acid into the CSF of the lateral ventricle can block the dipsogenic response

nfusions of hypertonic NaCl and induce a water diuresis in the nonhydrated animal out having other obvious effects. It provides additional evidence for the concept that ral receptors involved in the regulation of water intake and ADH secretion are in contact with the CSF and are susceptible to changes in its composition (Andersson

Since ethacrynic acid inhibits Na–K-ATPase (*cf* Schwartz *et al* 1975), the experi- s also lend support to the idea that active enzymatic cation transport forms an essential n the receptor excitation process. However a more indirect action of ethacrynic acid at the receptor level cannot be excluded as explanation for the inhibitory effects ob- d in the present study Choroidal Na–K-ATPase activity is a prerequisite for Na sport from the blood to the CSF (Vates *et al* 1974), and IVT infusions of a weak in- or of Na–K-ATPase (glycerol) have recently been observed to lower the CSF [Na] he goat much more effectively than corresponding glucose infusions (Olsson *et al.* 1976). the IVT infusions of ethacrynic acid might have lowered the [Na] of the CSF and nt in that manner indirectly have reduced the activity of juxtaventricular sodium sensitive ptors involved in the control of water balance. Therefore, it would be of interest to nd this rather preliminary study to involve also pre and post-infusion determinations of [Na⁺]

work was supported by the Swedish Medical Research Council (Project 04X-00500).

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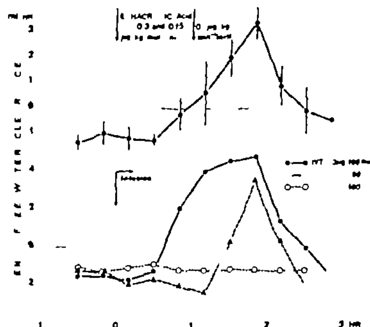


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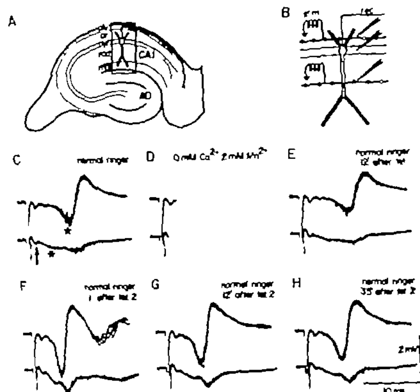


Fig. 1 A. Schematic drawing of the hippocampal slice with the studied region of CA1 framed. alv, alveus, or, stratum oriens, pyr, stratum pyramidale, rad, stratum radiatum, mol, stratum moleculare. B. Enlarged picture of the slice frame in A, showing positioning of stimulating (stim) and recording (rec) electrodes. C–H. Results from an experiment showing the field potentials evoked by stimulation of str. radiatum (commissural pathway). Each pair of traces consists of 5 superimposed recordings of potentials from str. pyramidale (upper trace) and str. radiatum (lower trace). C. Control, showing presynaptic volley (↑), field epsp (*), and population spike (★). D. Synaptic transmission blocked by 0 mM Ca^{++} 2 mM Mn^{++} recorded immediately before tetanus 1 (50 Hz in 5 s). E. Recovery of synaptic potentials 12 min after tetanus 1 showing absence of LLP, recorded immediately before tetanus 2 (50 Hz in 5 s). F, G, H. Potentiation of field epsp and population spike, recorded 1 min (F), 12 min (G), and 35 min (H) after tetanus 2. Although oscilloscope traces were photographed for only 35 min after the second tetanus on-line measurements showed that LLP persisted virtually unchanged for as long as the experiment was continued (duration of LLP > 2 h).

solution. After switching back to the normal fluid, but while synaptic transmission was still blocked, a tetanus was given (arrow tet 1). Within 15 min after the tetanus the responses of both sides recovered to their initial values, showing that no LLP had been produced. A second tetanus in normal solution (arrow tet 2) resulted in potentiation which outlasted the duration of the experiment (> 87 min). Results consistent with those described above were obtained in all of 5 expts. using calcium-free ringer and in 2 additional expts. using 1 mM Ca – 4 mM Mn to block synaptic transmission.

Measurements of the presynaptic volley during tetani showed that the amplitudes of the volley during intact and blocked synaptic transmission were essentially the same. This

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From the Department of Physiology University of Göteborg, Sweden
and the [1]Institute of Neurophysiology University of Oslo, Norway

Calcium dependency of synaptic long-lasting potentiation in the hippocampal slice

By

H WIGSTRÖM, J W SWANN and P ANDERSEN

Long lasting potentiation (LLP) of synaptic responses following short trains (tetar electric stimulation is an outstanding feature of the hippocampal formation (cf Bliss Lømo 1973 Schwartzkroin and Wester 1975) Little is still known about the mecha underlying LLP though increased transmitter output has been considered a likely possi (Andersen *et al* 1977). The results presented here demonstrate that the presence of e cellular calcium during the tetanus is necessary for the development of LLP

Experiments were performed on transverse hippocampal slices of guinea pigs (Skrede and Wes 1971). To reduce diffusion times a somewhat modified technique was developed in which entirely merged slices were used. The experimental situation is described in Fig. 1 A and B. T o synaptic to the CA1 pyramidal cells (stratum radiatum nd stratum oriens) were stimulated alternately potentials were recorded at the levels of the stimulati g electrodes and in the cell body layer (cf. Fig Presynaptic volley (†), field epsp (✳), and population spike (★) were measured.

The experiment began with a 15–30 min control period in normal perfusion solution (conta 2 mM Ca^{++}). Thereafter a sol tion with a modified composition was delivered producing a block synaptic transmission. The latter solution was usually calcium-free (in expts. combined ith addi Mg^{++} or Mn^{++}) but solutions with normal 2 mM calcium concentration containing 4 mM mang (calcium blocker) were also used Once synaptic transmission was blocked, tetanus (typically 50 Hz 5 s) was given t o e f the two synaptic nputs. Immediately thereafter or shortly before the tetanus perfusion solution was cha ged back to the normal. After synaptic transmission had recovered the pre presence of long lasting potentiation could be determined by comparing the amplitudes of field epsp population spike of the tetanized side w th the previous control values. Since LLP is an input-spe phenomenon (Andersen *et al* 1977) the responses evoked by stimulation of the non-tetanized side be used to verify that the preparation fully recovered from the cha ges in perfusion solution In to show that the slices were indeed ble t generate LLP a second teta us was given (in normal so and responses were monitored for 1 h thereafter

Results from one experiment are given in Fig. 1 C–H showing field potential record for the tetanized pathway The first tetanus given in a state of blocked synaptic transmis (0 mM Ca 2 mM Mn^{++}) produced no long-lasting potentiation (compare Fig. 2 C and while a subsequent tetanus in normal solution did (compare Fig. 2 E and G). The poten tion produced by the second tetanus remained virtually unchanged even after 2 h. T computer generated graphs in Fig. 2 show the time course of another experiment. P synaptic volley and population spike in response to oriens (tetanized pathway) and radiat (control) stimulation are plotted A calcium-free solution was used to block synaptic

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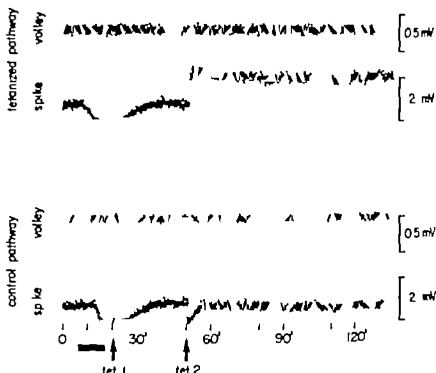


Fig 2. Presynaptic volley and population spike in response to stimulation of str. oriens (tetanized pathway, upper plots) and of str. radiatum (control pathway, lower plots). Bar indicates time for perfusion with 0 mM Ca^{++} solution. Arrows tet 1 and tet 2 mark the two tetani (50 Hz in 5 s, delivered to str. or. The stimulation strengths were finely adjusted by a computer in order to maintain constant presynaptic volley amplitudes.

ascertains that our results are not merely due to a failure of the volley to follow the tetanizing frequency during the synaptic blockade.

The results presented here show that the presence of calcium extracellularly—and most likely presynaptic calcium influx—is necessary during the tetanus in order to produce long-lasting potentiation (LLP). It may well be an indirect dependence, however, the mechanism being related to some link in the chain which starts with calcium entry and ends with the postsynaptic potential.

After this manuscript was prepared Dunwiddie *et al* (1978) published a report on a similar investigation. Our work in part confirms theirs but extends it since we measured not only population spike but also field epsp and presynaptic volley.

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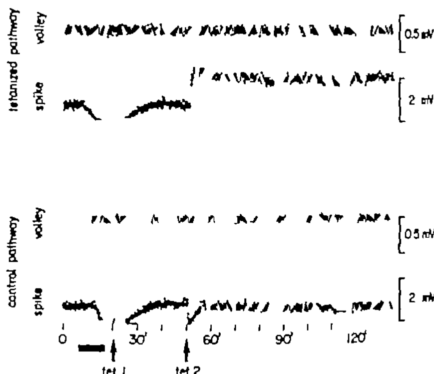


Fig 2. Presynaptic volley and population spike in response to stimulation of str oriens (tetanized pathway upper plots) and of str radiatum (control pathway lower plots). Bar indicates time for perfusion 0 mM Ca² solution Arrows tet 1 and tet 2 mark the two tetani (30 Hz in 5 s, delivered to str. or The stimulation strengths were finely adjusted by a computer in order to maintain constant presynaptic volley amplitudes.

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Demonstrations

Acta physiol. scand. 1979 105 129–136

o the Department of Physiology and Medical Biophysics, University of Uppsala, Sweden

A phenomenologic evaluation of CO_2-diffusion restriction in kidney tubules studied in an artificial membrane system

By

MORGAN SOHTELL

Received 25 April 1978

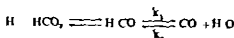
Abstract

HTELL, M. *A phenomenologic evaluation of CO_2-diffusion restriction in kidney tubules studied in an artificial membrane system.* Acta physiol. scand. 1979 *105* 129–136.

chemical course in multi-membrane system with interacting H and HCO_3^- ions has been described nomenologically as an analogy of the neutralization reaction between secreted H and filtered HCO_3^- in the proximal tubules of the kidney. It was shown that the produced CO_2 gave the highest P_{CO_2} so symmetrically placed reaction centre, which favours build-up of high intratubular P_{CO_2}. The CO_2 when is dependent on the rate-limiting permeation of the reacting ions, and the permeation could be ... ssed by the influence of solutions of macromolecules such as carbonic anhydrase, albumin and dextran.

Hydrogen ions are formed in the proximal tubular cells of the mammalian kidney and transported into the tubular lumen. Somewhere, the hydrogen ions react with the filtered bicarbonate ions, but the exact location of this neutralization reaction is unknown. It probably occurs in the neighbourhood of the luminal cell membrane. Carbon dioxide is formed and diffuses to the surroundings by its tension gradients.

The aim of this investigation was to describe phenomenologically the carbon dioxide diffusion out from the tubular lumen and how a build-up of a carbon dioxide difference between the tubular lumen and the tubular cell might occur. If hydrogen ions and bicarbonate ions are permitted to diffuse towards each other a reaction will take place as follows.



Carbonic acid will be formed, but as k is about 330 times greater than k (Garg *et al.* 1972) the equilibrium will be displaced to the right and carbon dioxide will be produced to a greater extent than carbonic acid. From that part of a system where the ions react with each other carbon dioxide will be formed, and carbon dioxide tension gradients will be built up.

Rector *et al.* (1965) and Vieira and Malnic (1968) compared the in vivo pH of the proximal tubular fluid with that obtained for the same fluid in vitro after equilibration to P_{CO_2} of

40 mmHg During control conditions this difference was small After carbonic anhydrase inhibition, however these mean differences ranged between 0 41 to 0 85 pH units. These were interpreted as a chemical nonequilibrium of the bicarbonate buffer system, induced by the enzyme inhibition Later experiments by Karlmark *et al* (1974), however pointed at the possibility of a higher P_{CO_2} in the tubular fluid than in the arterial blood. This was found to be quantitatively equivalent to the chemical non-equilibrium proposed. From direct measurements with a P_{CO_2} microelectrode, Sohtell *et al* (1976) obtained results which strongly favoured the assumption that the P_{CO_2} in the tubular fluid is higher than in the arterial blood.

The investigation was carried out in a multi-membrane system in which the hydrogen ion secretion and bicarbonate reabsorption were simulated by diffusion processes. It was then that the rate of transport of the carbon dioxide could be altered

Material and Methods

In order to study the reaction between H and HCO coming into a membrane from either side, the membrane was regarded as being divided into nine slices. A multi-chamber system was thus developed, in which the chambers were separated by cellophane membranes. The complete equipment (see Fig 1) consisted of 10 thermostated chambers. The two end chambers were each connected to a glass container (1 liter) reservoirs I and II (Fig. 1) via a pump. The end chamber of the hydrogen side was No. 1 and that of the bicarbonate side No 10. The membrane between chambers 1 and was called membrane No. 1 and so forth. Stirring mechanisms driven by electromagnet and silver silver chloride electrodes for potential difference recordings were situated in the chambers (Fig). In the present investigation the potential differences were recorded so as to be able to follow from outside the chambers the development of a steady state in the reaction system. The electrodes were connected to a mV meter (PHM26, Radiometer Copenhagen, Denmark). The experiments were performed at 25°C.

The membranes were cut from cellophane tubings (C 75 Dialysis membrane Union Carbide Corporation, Chicago Illinois 60638) When studying events taking place in membranes, the influence of the surrounding unstirred layers has to be considered The effective layer connected to smooth cellophane membranes is not reduced below the order of 0 03 mm, even with vigorous stirring. The thickness of the un-

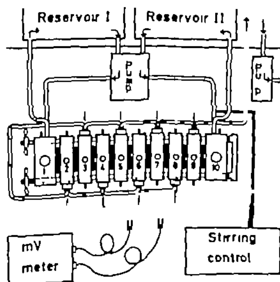
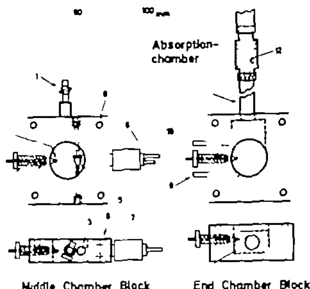


Fig. 1 The complete equipment showing chambers mounted on to a guide stand pressed together with wingnuts, the two reservoirs in the thermostated water bath, the pump circulating thermostated water and end chambers solutions, respectively the stirring electromagnets the mV-meter



2 The chamber blocks: 1) stopcock,) Ag/AgCl electrode, 3) electrodeholder 4) O-rings, 5) propeller ith its spider and built-in rod magnet, 6) iron-core ith channels for thermostated ater ed, 8) holes for the guide stand, 9) and 10) inlet and outlet for the circulating end-chamber solution, 11) setting tube, 12) container for the barium-dihydroxide solution.

as therefore has to exceed 0.2 to 0 8 mm to minimize the influence of the unstirred layers (Teorell 1936). his investigation the membranes were made about 0 7 mm thick (wet thickness), through superimposi- of three pieces of the above-mentioned tubings (Teorell 1936).

The end chamber concentrations of H and HCO_3 in this model experiment were so selected as to give ces hose relations ere approximately comparable to the prevailing conditions in the proximal tubules he kidney 1 order to evaluate the relative chemical forces (F) of the hydrogen ion secretion and the bi- bonate ion reabsorption, respectively in the proximal tubules, the following equation and values were d

$$F \quad A \log \frac{C_{lumen}}{C_{cell}}$$

ere A proportionality constant and C represents the concentration of the ion species. (H^+) lumen – 10 M (Karlmark *et al* 1974), cell 8 9 10^{-8} M (Waddel *et al* 1969). (HCO_3^-) lumen = 25 10^{-3} M probable concentration of the glomerular filtrate in Bowman's space, without Donnan factor and plasma ter corrections, the plasma concentration), cell 11 1 10^{-3} M (Khuri *et al.* 1974). This resulted in ative driving force for bicarbonate ions of about 0 35 and for hydrogen ions of 0 35.

In the first experiment the following solutions were deposited in the respective chambers. No. 1 100 mM l, Nos 2 9 5 mM NaCl and No 10 95 mM $NaHCO_3$ + 5 mM NaCl. Chloride was placed in all chambers that potential changes could be followed from the beginning

In the second experiment the catalytic effect of three kinds of macromolecules on the reaction system as died The macromolecules were dissolved in 5 mM NaCl solution to equal final concentrations The lowing solutions were studied. 0 05 mM human carbonic anhydrase type B (HCAB), 0 05 mM human umin (AB Kabi, Stockholm, Sweden) and 0 05 mM dextran (Dextran T_{40}, Pharmacia Fine Chemicals, psala, Sweden) These solutions ere deposited in chambers 2-9 The concentration of carbonic an- drase as about fi times that in the kidney cell (Maren 1969).

Kindly received from Dr P Wistrand, Department of Pharmacology Uppsala University Uppsala, Sweden

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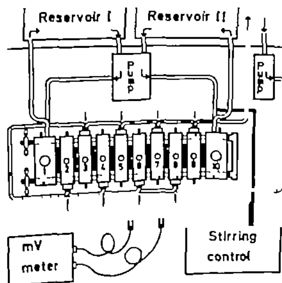
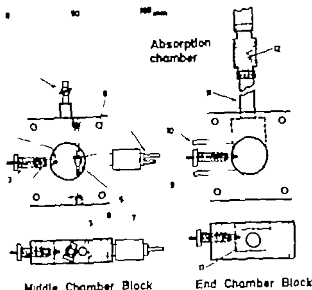


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$$F = A \log \frac{C_{lumen}}{C_{cell}}$$

[illegible] A [illegible] proportionality constant and C represents the concentration of the ion species. (H^+) lumen = [illegible] 10^{-8} M (Karlmark et al 1974), cell [illegible] 8.9 [illegible] 10^{-8} M (Waddell et al 1969). (HCO_3^-) lumen = 25 [illegible] 10^{-3} M [illegible] concentration of the glomerular filtrate in Bowman's space, [illegible] Donnan factor and plasma [illegible] corrections, i.e. the plasma concentration), cell [illegible] 11.1 [illegible] 10^{-3} M (Khuri et al. 1974). This resulted in [illegible] driving force for bicarbonate ions of about 0.35 and for hydrogen ions of −0.35.

In the first experiment the following solutions were deposited in the respective chambers: No. 1 100 mM [illegible], Nos 2-9 5 mM NaCl and No 10 95 mM $NaHCO_3$ [illegible] 5 mM NaCl. Chloride was placed in all chambers [illegible] that potential changes could be followed from the beginning.

In the second experiment the catalytic effect of three kinds of macromolecules on the reaction system [illegible] [illegible]. The macromolecules [illegible] are dissolved in 5 mM NaCl solution to equal final concentrations. The [illegible] solutions were studied: 0.05 mM human carbonic anhydrase type B (HCAB), 0.05 mM human [illegible] (AB Kabi, Stockholm, S[illegible]eden) and 0.05 mM dextran (Dextran $T_{[illegible]}$, Pharmacia Fine Chemicals, [illegible], Sweden). These solutions [illegible] are deposited in chambers 2-9. The concentration of carbonic an[illegible] [illegible] is about fi[illegible] times that in the kidney cell (Maren 1969).

[illegible] Kindly received from Dr P. W[illegible], Department of Pharmacology, Uppsala University, Uppsala, [illegible]

In the *third* experiment the solutions were the same as in the first and second expts. The transpor carbon dioxide to chambers 1 and 10 was studied as the rate of carbon dioxide liberation from these chambers. The released carbon dioxide was absorbed and titrated by a barium-dihydroxide solution with bromthymol blue as an indicator. The solution was kept in a small cup situated in the absorption chamber, cf. in Fig. 2.

Analyses of about 500 μl of the content of each chamber were performed by the following methods

(H) Hydroxyl titration with brom-thymol-blue as indicator performed in a micro-titration (Öbrink 1955).

(Cl^-) Micro-chloride titration according to the method of Ramsay (Ramsay *et al.* 1955).

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P_{CO_2} Pco measurements were performed with a P_{CO_2} electrode (E5036, Radiometer, Copenhagen, Denmark) connected to a mV-meter (PHM26, Radiometer)

Results

Fig. 3 shows the ion concentrations and the carbon dioxide tension profiles in the first experiment. The results are considered to have been obtained under a relatively steady state condition (after 5 days). According to the carbon dioxide profiles the neutralisation reaction between H and HCO_3 took place somewhere in the eighth membrane. The quotient between the P_{CO_2} gradients from the reaction centre will be equal to the P_{CO_2} quotient between the outermost membranes, as the beginnings of the gradients are the same. The P_{CO_2} in a membrane was calculated as the mean P_{CO_2} of the adjacent chambers. The P_{CO_2} in membrane and 9 were 11.0 and 87.? mmHg, respectively which will give a quotient of 7.9

In the *second* experiment where chambers 2 to 9 contained solutions of HCAB, albumin and dextran, a good steady state was obtained. The P_{CO_2} at the reaction centre, the p

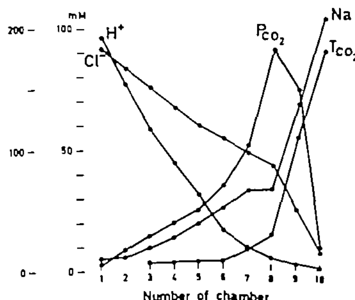
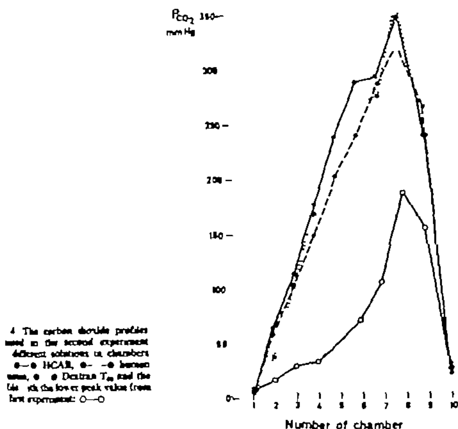


Fig. 3 The results in the first experiment obtained after a diffusion time of five days. T_{CO_2} represents the total carbonic acid content



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...LE I. The liberation rates of carbon dioxide and the quotients of the liberation rates between chambers 10 and 1 as obtained from the third experiment. These quotients are to be compared ...ith the quotients for the carbon dioxide tension gradients in the first and second experiments.

Solutions	CO_2 liberation/min		
...mber Nos. 2 9	No. 1	No. 10	Chamber No. 10/1
...M NaCl	1.0	9.3	9.3
...M NaCl albumin	6.0	17	3.6
...M NaCl HCAB	10.4	31.4	3.0
...M NaCl dextran	7.5	20.3	2.7

In the *third* experiment the solutions were the same as in the first and second expts. The transp carbon dioxide to chambers 1 and 10 was studied as the rate of carbon dioxide liberation from bers. The released carbon dio ide was absorbed and titrated by a barium-dihydroxide solution with thymol-blue as an indicator The solution was kept in a small cup situated in the absorption chamber, ε In Fig. ..

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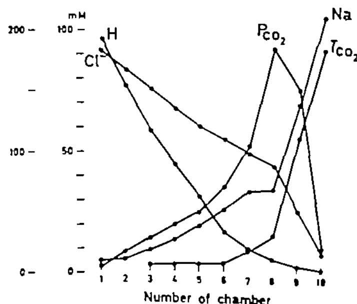
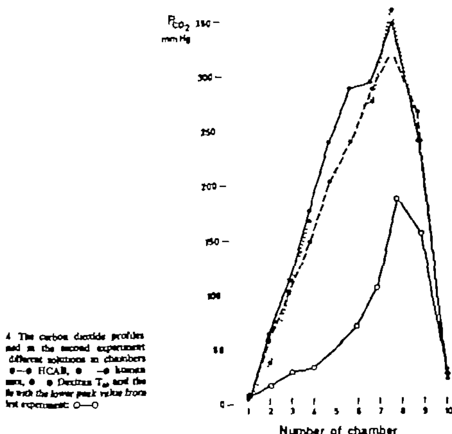


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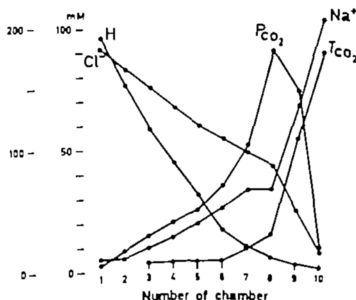
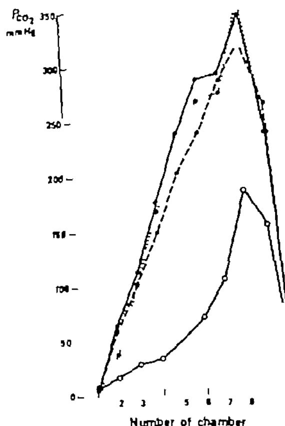


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lution		CO_2 liberation/time		
mber Nos. 2-9		No 1	No. 10	Chamber No. 10/1
M NaCl		1.0	9 3	9 3
mM NaCl	albumin	6 0	21.7	3.6
mM NaCl	HCAB	10.4	31.4	3 0
mM NaCl	dextran	7 5	20 3	2 7

Discussion

In a system with several chambers in series, Teorell (1936) studied the distribution of ... a membrane made up of the chamber-separating membranes. The ions were found to be distributed according to their mobilities and concentration differences in the chamber sys... He also studied the effect on ion distribution of a chemical reaction within the cha... system From the distribution and the effects of concentration differences in the cons... system the location of the reaction can be calculated. The concentrations of hydrogen ... bicarbonate ions are assumed to be equal in either end of the system. The relative mobi... of these ions are 350 and 43.5 respectively at 25 C (Handbook of Chemistry and Phy... 1971) If the length of the system is set at 1 0 and the mobilities of the reacting ions as... sumed not to be influenced by other ions, the reaction location will occur 0.11/1 of the d... tance from the side where bicarbonate emanates. In steady state the concentration prof... are linear to the point of reaction, which then means that the carbon dioxide concentra... gradient and thus the rate of carbon dioxide transport towards the bicarbonate side is th... retically 8 0 times greater than that towards the hydrogen side This value is in accord... with the result of 7 9 obtained in the first experiment, thus also verifying the asymmetr... located reaction centre

A number of reports have described a facilitation of carbon dioxide transport thro... different boundaries The concentrations of the reacting ions in the different chamber co... partments in the present investigation are considered to be homogenous, but not at t... membranes and their connecting unstirred layers. Gutknecht *et al* (1977) found that carb... dioxide diffusion can be rate limiting for total carbon dioxide transport in lipid bilayers ... unstirred layers, as the neutralisation reaction is too slow to permit even a high bicarbo... concentration to speed up the carbon dioxide production more than 15%. In carbo... anhydrase-catalyzed reactions they found that the bicarbonate ion mobility in itself would... rate-limiting. This supports the finding of a lower peak P_{CO_2} in the case of the uncatal... reaction as compared with the catalyzed one, which thus could be an effect of a slo... bicarbonate permeation through the unstirred layer Suchdeo *et al* (1974) found a 3 to... fold increase of the carbon dioxide flux through a Gelman membrane when adding carbo... anhydrase, and Enns (1967) reported a doubled transport rate through a Millipore filter ... washed red-cell ghosts or filtrate from the red-cell solution deposited on one side of ... membrane

The relative resistance to permeation of the reacting ions in the unstirred layers can ... decreased by co-transport in translational diffusion of macromolecules. Gros *et al* (19... observed that thin layers of albumin solutions facilitated hydrogen ion diffusion and ... also found greater permeation of bicarbonate ions, which was explained as a secondary e... of the increased hydrogen ion diffusion. The facilitating effect of the dextran solution is ... fully understood. The results from the second experiment, in which the macromolecular s... tions were used, showed a tendency to enhanced transport of carbon dioxide towards ... hydrogen and bicarbonate ion side, indicated by the increased P_{CO_2} gradients compared ... the first experiment. This facilitation was demonstrated by the carbon dioxide libera... study in the third experiment.

Recently Wistrand *et al.* (1977) showed the existence of an enzyme that is kinetically similar HCAB in the brush border and peritubular membranes of the rat kidney The membrane [illegible]yme as found to have 3% of the total cell enzyme activity If the distribution of proteins [illegible]de the cell is equal to that in the membranes, this should lead to a somewhat higher con[illegible]tration in the membranes. According to Maren (1969), however only 0 1% of the enzyme [illegible]ply in the tubular tissue is utilized. The amount of carbonic anhydrase in the present [illegible]estigation is therefore regarded as being in a suitable excess.

The increasing effect of carbonic anhydrase on the reaction rate is, however of minor [illegible]portance in the present system. As shown in Fig 4, all the macromolecules studied give [illegible]out the same carbon dioxide profile. This means that the macromolecular reduction of the [illegible]stance to permeation of the reacting ions is of the same order The permeation of the ions [illegible]s seems to be rate-limiting for carbon dioxide transport. The effect of the carbonic an[illegible]drase on the reaction rate would probably have been more pronounced if the carbon dioxide [illegible]nsport had been rate-limiting in this membrane system. The driving force for carbon [illegible]xide diffusion through a membrane should be increased by an enzymatic conversion of [illegible]rbon dioxide into bicarbonate.

The phenomenologically described chemical course in this investigation on interacting [illegible]drogen and bicarbonate ions could be analogous to the situation in the proximal tubules. [illegible]e finding of the elevated P_{CO_2} in the proximal tubules (Schtell *et al.* 1976) may be a result [illegible] a neutralization reaction taking place adjacent to the luminal cell membrane. The elevated [illegible]$_{O_2}$ in the reaction centre would create an increased luminal fluid P_{CO_2} and a build-up of a [illegible]adient for carbon dioxide transport out from the tubular lumen. The results also indicate [illegible]at the net carbon dioxide transport is enhanced by carbonic anhydrase, by its increasing [illegible]fect on the permeation of hydrogen and bicarbonate ions.

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P_{CO_2} of the proximal tubular fluid and the efferent arteriolar blood in the rat kidney

By

MORGAN SOHTELL

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Abstract

HTELL, M. *P_{CO_2} of the proximal tubular fluid and the efferent arteriolar blood in the rat kidney* Acta physiol. scand. 1979 *105* 137 145

cordings in vivo of the carbon dioxide tension of the proximal tubular fluid and of the efferent arteriolar od were performed with P_{CO_2} microelectrodes in the rat kidney The buffer lines of the efferent arteriolar od and systemic arterial blood were determined with an ultramicro equilibration system and the acid- e status of the systemic arterial blood as measured. The intratubular P_{CO_2} as significantly higher than P_{CO_2} of the arterial blood, and the P_{CO_2} of the efferent arteriolar blood as significantly lower than that the arterial blood. The buffer capacity was higher and the bicarbonate concentration slightly lower for efferent arteriolar blood than for the arterial blood. It is concluded that P_{CO_2} difference exists across tubular all and that the high intratubular P_{CO_2} favours chemical equilibrium of the carbonic acid- carbonate system in the proximal tubular fluid It is supposed that the slightly lowered bicarbonate con- ntration in the efferent arteriolar blood is an effect of the glomerular ultrafiltration process.

y the ultrafiltration process in the kidney the proximal tubules are offered a load of bicarbo- ate. The mechanisms underlying the tubular bicarbonate reabsorption have been the subject much discussion (see Malnic and Giebisch 1976). One of these mechanisms generally voured is the active hydrogen ion transport into the tubular lumen. According to this hypo- esis the H and HCO_3 ions react with one another and form carbonic acid. This acid is then ehydrated, forming water and carbon dioxide, which are then reabsorbed. The rate of this eabsorption may be influenced by various factors, such as the rate of delivery of H the ehydration rate and the carbon dioxide diffusion across the tubular wall.

Rector et al (1965) and Vieira and Malnic (1968) made direct recordings of the intra ubular pH with micropuncture techniques. They measured the pH of urine samples taken rom the same puncture site and equilibrated to a P_{CO_2} of 40 mmHg, and found the in situ pH o be lower than the pH of the equilibrated samples. It was concluded that the lower in situ pH was due to a lower rate of dehydration of the carbonic acid, and it was thus considered to epresent chemical non-equilibrium state.

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Karlmark and Danielson (1974) also made in situ pH measurements but equilibrated the rine samples from the proximal tubules to CO of different concentrations. They were then

able to calculate the actual P_{CO_2} of the tubular urine. This was found to be higher than P_{CO_2} of the arterial blood and they concluded that a chemical equilibrium existed with regard to the dehydration of the carbonic acid, but a non-equilibrium concentration of P_{CO_2} across the tubular membrane

With the aim of contributing to the discussion of whether the dehydration represents a chemical non-equilibrium or equilibrium state, direct P_{CO_2} measurements were made with P_{CO_2} microelectrodes in this investigation

With the technique used it was also possible to eavaluate the acid-base conditions of blood of the efferent arterioles and thus calculate the difference in P_{CO_2} between proximal tubular urine and the blood of the early peritubular capillaries.

Material and Methods

Male albino rats (Sprague Dawley strain A Ucimex, Stockholm, S eden) weighing 225 to 3.5 grams on a diet of commercial rat pellets (Anticimex, Sweden) were used and were allowed food and water ad lib until the start of the experiment. They were naesthetized by an i traperitoneal injection of Inactin (Fabr Promonta GmbH Hamburg, W Germany) in a dose of 120 mg/kg body weight (b.w.).

The body temperature was kept at 37.5 C with servo-controlled heating table The rats were tracheotomized and a catheter w s inserted into the left femoral ein and left femoral artery for infusions and blood pressure recording, respectively After catheterization a continuous intra enous (i.v.) infusion of 3 ml b.w nd hou of a Ri ger solution w started. The urinary bladder was drained to avoid diminished flow (Karlmark nd Danielson 1974) The left kidney was reached through a flank incision and was mobilized i locit cup by embedding it in 2 ga in-Ri ger solution. The left ureter as catheterized and the exposed dorsal surface of the kidney was superfused with warm liquid paraffin, pre-equilibrated with a mi ture of 5 CO_2 and 70 O in nitrogen

Experimental procedure For the biological experiments the rats were divided into two groups.

In group I the P_{CO_2} microelectrode was used for ch determinations of the carbon dioxide tension in the convoluted proximal tubules and i the superficial efferent arterioles (welling points) The proximal tubules in estigated were chosen randomly Arterial blood was sampled simultaneously for determination of the acid-base status according to the method of Siggaard Andersen (1967).

The rats of group II were used for measurement of the acid-base condition of welling point blood micro technique described below 15 min before the blood was collected, the rats were given an i injection of 2 000 IU/kg b w of a heparin solut on (Vitrum, Stockholm, Sweden) t avoid clotting of the blood. The sampling pipettes were pulled and ground in a similar manner to the o termost capillary of the P_{CO_2} electrode (see below) The tip was given diameter of about 12 µm. The sampling pipette as filled with coloured liquid paraffin. The blood flowed to the pipette by its own pressure. Arterial blood as collected for nalyses in the Astrup pparatus

The P_{CO_2} microelectrode The main principle of this electrode is w ll k own (Severinghaus and Bradley 1958). It has previously been described by Sohtell nd Karlmark (1976) and brief description is given as following. A ordinary Pyrex glass tube (inner diameter 1 0 mm, outer diameter 1 4 mm) is pulled to a diameter of a few µm and ground angularly t permit gentle puncture. An ant mony-Ag AgCl electrode system is made and introduced together with second capillary into the ground glass tube, as shown in F 1 A third glass capillary is mou ted n the rear end and all components are glued together The lead of electrodes leave the unit through the glue drop A solution containi g 145 mM N Cl and 5 mM NaHCO is aspirated into the electrode through th tip The electrode potential is then measured and must have empirically accepted value, i.e. 500 ± 15 mV The tip is finally pl gged by d pping it nto a drop of silicone rubber (Elastosil, Wacker-Chemie, GmbH, Munchen, W Germany). This special rubber is used because requires moisture for curing, which assures tight plug.

The electrode is calibrated before and after each measurement device designed as a block of cups with nozzels in the bottom through which high gas flows give well-defined pressures of CO_2. The calibration cups are shown in F g. close to kidney cup. The calibration gas is thermostated t the same temperat re as that of the tissue. The calibration and measuri period of three minutes respectively according t Sohtell and K

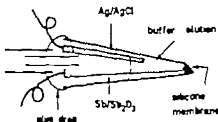


Fig. 1

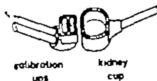


Fig. 2

1 The P_{CO_2} electrode. The three glass capillaries are connected to each other as well as to the pH trode system by the same drop of glue (epoxy resin).

2 The thermostated calibration cups and the kidney cup as arranged for tissue studies.

Acid-base status of the rat This as determined as the acid-base status of the arterial blood by the Astrup ibration technique as described by Siggaard Andersen (1967), using the nomogram for man, hich can used for animals too. The normal values, compared to man, are different, however

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The electrode unit then transferred to beaker containing an equilibration gas mixture of 3 CO_2 in gen A 10 to 15 nl blood sample is deposited on the glass-cup microelectrode and the sample droplet is ntacted ith Ag AgCl reference electrode The pH is measured and the procedure is then repeated in aker content equilibrated with 12 CO_2 in oxygen

I the present investigation this equilibration ultramicro method was standardized with the arterial blood uilibrated in the Astrup apparatus. The buffer lines of the arterial blood obtained with the tw methods ere parallel Those obtained ith the micro unit ere, however bout 0 04 pH units more acid than the ues from the Astrup apparatus This is probably due to protein error of the glass-cup microelectrode in e equilibration micro-unit.

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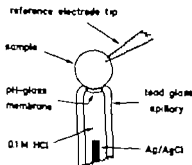


Fig 3. The tips of the electrodes as arranged in the equilibration ultramicro unit. The drawing also show sample droplet on the pH-glass membrane designed as cup.

able to calculate the actual P_{CO_2} of the tubular urine This was found to be higher than P_{CO_2} of the arterial blood and they concluded that a chemical equilibrium existed with to the dehydration of the carbonic acid, but a non-equilibrium concentration of P_{CO_2} ... the tubular membrane

With the aim of contributing to the discussion of whether the dehydration represents chemical non-equilibrium or equilibrium state direct P_{CO_2} measurements were made P_{CO_2} microelectrodes in this investigation.

With the technique used it was also possible to eavaluate the acid-base condition of blood of the efferent arterioles and thus calculate the difference in P_{CO_2} between proximal tubular urine and the blood of the early peritubular capillaries.

Material and Methods

Male albino rats (Sprague-Dawley strain Anticimex, Stockholm, Sweden) weighing 225 to 325 gram fed a diet of commercial rat pellets (Anticimex, Sweden) were used and were allowed food and water ad lib until the start of the experiment. They were anaesthetized by an intraperitoneal injection of Inactin (Fa F br Promonta, GmbH Hamburg, W Germany) n dose of 170 mg/kg body weight (b.).

The body temperature was kept at 37.5 C with a servo-controlled heating table. The rats were tracheotomized and a catheter was inserted into the left femoral vein and left femoral artery for infusions and blood pressure recording, respectively After catheterization a continuous intravenous (i) infusion of 10 ml b.w and hour of Ringer solution was started. The urinary bladder was drained to avoid diminished urine flow (Karlmark and Danielson 1974) The left kidney was reached through a flank incision and was immobilized in a lucite cup by embedding it in a agar-in-Ringer solution. The left ureter was catheterized nd the exposed dorsal surface of the kidney w s superfused w th warm liquid paraffin, pre-equilibrated with a mixture of 5 CO_2 and 20 O_2 in nitrogen.

Experimental procedure For the biological experiments the rats were divided into two groups.

I group I the P_{CO_2} microelectrode was used for *vivo* determinations of the carbon dioxide tension the convoluted proximal tubules nd in the superficial efferent arterioles (welling points) The proximal tubules invest gated were chosen randomly Arterial blood was sampled simultaneously for determination the acid-base status according to the method of Siggaard Andersen (1967).

The rats of group II were used for measurement of the acid-base condition of welling point blood with micro technique described below 15 min before the blood was collected, the rats were given an injection of 000 IU/kg b.w of a heparin solution (Vitrum, Stockholm, Sweden) to avoid clotting of the blood. The sampling pipettes were pulled and ground in a similar manner t the outermost capillary of the P_{CO_2} electrode (see below) The tip was given a diameter of about 12 μm. The sampling pipette was filled with coloured liquid paraff n. The blood flowed into the pipette by its own pressure. Arterial blood was collected for analyses in the Astrup apparatus.

The P_{CO_2} microelectrode The main principle of this electrode is well known (Severinghaus and Bradley 1958). It has previously been described by Sohtell and Karlmark (1976) and brief description is given in the following. An ordinary Pyrex glass tube (inner diameter 1.0 mm, outer diameter 1 4 mm) is pulled to a diameter of few μm and ground angularly to permit a gentle puncture. An antimony Ag AgCl electrode system is made and introduced together with a second capillary int the ground glass tube, as shown in Fig. 1 A third glass capillary is mounted in the rear end and all components are glued together The wires of electrodes lea e the unit through the glue drop. A solution containing 145 mM NaCl and 5 mM NaHCO3 is aspirated into the electrode through the tip. The electrode potential is then measured and must have an empirically accepted value, i.e. 500 ± 15 mV The tip is finally plugged by dipping it into drop of silicone rubber (Elastosil, Wacker-Chemie, GmbH, München, W Germany). This special rubber is used because it requires moisture for curing, which assures tight plug.

The electrode is calibrated before and after each measurement in device designed as block of cups with nozzels in the bottom through which high gas flows give well-defined pressures of carbon dioxide The calibration cups are shown in Fig. close to kidney cup. The calibration gas is humidified and thermostated to the same temperature as that of the tissue. The calibration and measuring time is always period of three minutes respectively according to Sohtell and Karlmark (1976).

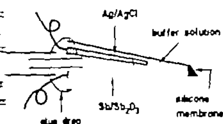


Fig. 1

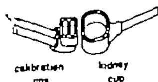


Fig. 2

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The electrode unit is then transferred to a beaker containing an equilibration gas mixture of 3 % CO_2 in oxygen. A 10 to 15 nl blood sample is deposited on the glass-cup microelectrode and the sample droplet is contacted with the Ag/AgCl reference electrode. The pH is measured and the procedure is then repeated in a beaker content equilibrated with 1 % CO_2 in oxygen.

In the present investigation this equilibration ultramicro method was standardized with the arterial blood equilibrated in the Astrup apparatus. The buffer lines of the arterial blood obtained with the two methods were parallel. Those obtained with the micro unit were, however, about 0.04 pH units more acid than the ones from the Astrup apparatus. This is probably due to a protein error of the glass-cup microelectrode in the equilibration micro-unit.

The slopes of the buffer lines were evaluated in the Siggaard Andersen nomogram as follows. The relation between the buffer lines of capillary and arterial blood was calculated after the analyses of blood in the ultramicro equilibration unit. This relation was transferred to the buffer line of the arterial blood, analyzed in the Astrup apparatus and plotted in the nomogram. A true representation of the buffer lines was thus obtained.

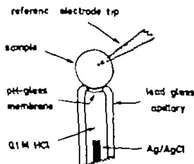


Fig. 3. The tips of the electrodes as arranged in the equilibration ultramicro unit. The drawing also shows a sample droplet on the pH-glass membrane designed as a cup.

able to calculate the actual P_{CO_2} of the tubular urine. This was found to be higher the P_{CO_2} of the arterial blood and they concluded that a chemical equilibrium existed with re[illegible] to the dehydration of the carbonic acid, but a non-equilibrium concentration of P_{CO_2} [illegible] the tubular membrane

With the aim of contributing to the discussion of whether the dehydration repre[illegible] chemical non-equilibrium or equilibrium state, direct P_{CO_2} measurements were made [illegible] P_{CO_2} microelectrodes in this investigation.

With the technique used it was also possible to eavaluate the acid-base conditions of [illegible] blood of the efferent arterioles and thus calculate the difference in P_{CO_2} between [illegible] tubular urine and the blood of the early peritubular capillaries.

Material and Methods

M[illegible]le albino rats (Sprague Dawley strain Anticimex, Stockholm, S[illegible]eden) weighing 225 to 325 grams [illegible] a diet of commercial rat pellets (Anticimex, Sweden) were used and were allowed food and water [illegible] until the start of the experiment. They were anaesthetized by an intraperitoneal injection of Inactin® [illegible] Fabr Promonta GmbH Hamburg, W Germany) in a dose of 120 mg/kg body weight (b[illegible])

The body temperature w[illegible]s kept at 37.5 C with a servo-controlled heating table. The rats [illegible] mized and a catheter was inserted into the left femoral [illegible]ein and left femoral artery for infusions and [illegible] pressure recording, respectively After catheterization a continuous intra[illegible]enous (Lv) infusion of 3 [illegible] b.w [illegible]nd hour of a Ringer solution was started The uri[illegible]ary bladder was drained to avoid diminished [illegible] flow (Karlmark and Danielson 1974). The left kidney was reached through a flank incision and was mobilized in a lucite cup by embedding it in a [illegible]gar-in-Ringer solution. The left ureter [illegible] and the [illegible]posed dorsal surface of the kidney was superfused with warm liquid paraffin, pre-equilibrated w[illegible]th a m[illegible]ture of 5 [illegible] CO_2 and 90 [illegible] O in nitrogen

Experimental procedure For the biological experiments the rats were divided into two groups.

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The electrode is calibrated before [illegible]nd after each measurement in [illegible] device designed as a [illegible] cups with nozzels in the bottom through which high gas flows give well-defined pressures of carbo[illegible] The calibration cups are shown in Fig. 2 close to [illegible] kidney cup. The calibration gas is humidifie[illegible] thermostated to the same temperature as that of the tissue. The calibratio[illegible] and measuring time is period of three minutes respectively according to Sohtell and K[illegible]rlmark (1976).

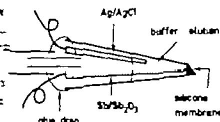


Fig. 1

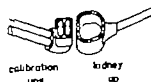


Fig. 2

1 The P_{CO_2} electrode. The three glass capillaries are connected to each other as well as to the pH rode system by the same drop of glue (epoxy resin)

2 The thermostated calibration cups and the kidney cup as arranged for these studies.

Acid-base status of the rat. This was determined as the acid-base status of the arterial blood by the Astrup calibration technique as described by Siggaard Andersen (1967), using the nomogram for man, which can be used for animals too. The normal values, compared to man, are different, however.

Determination of the buffer line of µl blood samples. The analyses were performed in an equilibration device described by Karlmark and Sohtell (1973). This consists of a closed thermostatted water jacket surrounding beakers containing liquid paraffin. The liquid paraffin in each beaker can be equilibrated individually with gas mixtures through a nozzle in the bottom of the beaker. The antimony electrode system used for the measurements originally described was exchanged, however, for a glass-cup microelectrode (Karlmark et al. 1971). A schematic drawing of the electrode arrangement is given in Fig. 3. The electrode is calibrated with phosphate buffer solutions in a beaker containing water-saturated liquid equilibrated with air. The electrode unit is then transferred to a beaker containing an equilibration gas mixture of 3% CO_2 in oxygen. A 10 to 15 nl blood sample is deposited on the glass-cup microelectrode and the sample droplet is contacted with the Ag/AgCl reference electrode. The pH is measured and the procedure is then repeated in another container equilibrated with 12% CO_2 in oxygen.

In the present investigation this equilibration ultramicro method was standardized with the arterial blood calibrated in the Astrup apparatus. The buffer lines of the arterial blood obtained with the two methods were parallel. Those obtained with the micro unit were, however, about 0.04 pH units more acid than the values from the Astrup apparatus. This is probably due to a protein error of the glass-cup microelectrode in the equilibration micro-unit.

The slopes of the buffer lines were evaluated in the Siggaard Andersen nomogram as follows. The relation between the buffer lines of a ... point and arterial blood was calculated after the analyses of blood in the ultramicro equilibration unit. This relation was transferred to the buffer line of the arterial blood, analysed in the Astrup apparatus and plotted in the nomogram. A true representation of the buffer lines was thus obtained.

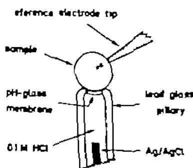


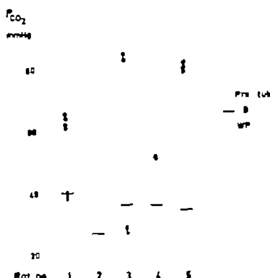
Fig. 3. The tips of the electrodes as arranged in the equilibration ultramicro unit. The drawing also shows the sample droplet on the pH-glass membrane designed as a cup.

TABLE I. The acid-base parameters from the first group of rats. AB = arterial blood, WP [illegible] blood, TUB = tubular fluid. As = Astrup apparatus. El = microelectrode.

Rat	Sample	pH	P_{CO_2} (As) mmHg	HCO_3^- mM	Base excess mM	P_{CO_2} (El) mmHg
1	AB 1	7.39	40.0	23.9	−0.2	
	TUB 1					65.7
	WP 1					39.3
	TUB 2					53.5
	TUB 3					61.1
	TUB 4					56.6
	TUB 5					64.2
	TUB 6					62.3
	WP 2					38.3
	WP 3					40.1
	TUB 7					53.2
	WP 4					40.2
	AB 1	7.49	27.0	20.8	−1.0	
	TUB 1					40.4
	TUB 2					42.4
	TUB 3					46.1
3	AB 1	7.44	36.2	23.8	+0.6	
	WP 1					28.5
	TUB 1					74.5
	WP 2					27.4
	TUB 2					41.9
	TUB 3					77.6
	TUB 4					84.9
	TUB 5					82.5
	WP 3					29.0
4	AB 1	7.50	36.1	28.0	+5.1	
	TUB 1					63.7
	TUB 2					51.2
	TUB 3					48.1
	TUB 4					61.1
	TUB 5					57.4
	TUB 6					64.2
	WP 1					30.8
	TUB 7					42.4
	TUB 8					51.8
5	AB 1	7.43	34.6	22.8	−0.9	
	TUB 1					44.0
	WP 1					30.0
	TUB 2					55.4
	TUB					51.8
	TUB 4					64.7
	TUB 5					61.1
	TUB 6					78.7
	TUB 7					74.5
	TUB 8					81.8
	TUB 9					79.7

Results

Acid-base status and P_{CO_2}. The values for the acid base parameters of the arter tained in the first group of rats are given in Table I. Fig. 4 shows the P_{CO_2} val the proximal tubular lumen and in the welling points with the P_{CO_2} microele



4 The results of the P_{CO_2} measurements in the rats of group I. AB arterial blood, WP welling point blood.

the tubular urine was always higher than that of the arterial blood taken in connection with the tubular measurements (P 0.001).

In four of the five rats the P_{CO_2} measurements in the welling points were successful. These showed a slightly lower P_{CO_2} in the welling point blood than in the arterial blood ($P < 0.01$)

Buffer lines. In the second group of rats, the welling point blood and arterial blood were compared with respect to their buffer properties. The Siggaard Andersen nomogram for

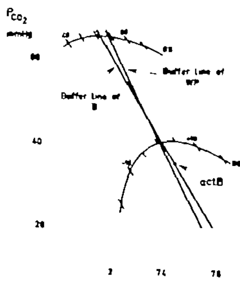


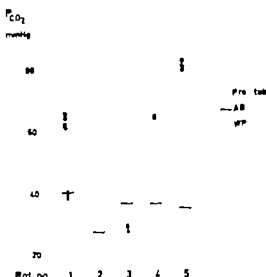
Fig. 5 The buffer lines obtained from rat 2 in group II. The actual pH and the corresponding P_{CO_2} obtained for the arterial blood are shown on the buffer line by the point actB. AB and WP are the arterial and welling point blood, respectively. BB and BE are the scales in mM concentrations for buffer base and base excess, respectively. The diagram is redrawn from the Siggaard Andersen curve nomogram (Radiometer, Copenhagen, Denmark).

TABLE I The acid-base parameters from the first group of rats. AB = arterial blood, WP [illegible] blood, TUB = tubular fluid, As = Astrup apparatus. El = microelectrode.

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	WP 1					30.0
	TUB 2					55.4
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Results

Acid-base status and P_{CO_2}. The values for the acid base parameters of the arterial blood ob-tained in the first group of rats are given in Table I Fig. 4 shows the P_{CO_2} values obtained i[n] the proximal tubular lumen and in the welling points with the P_{CO_2} microelectrodes. The P_{CO_2}

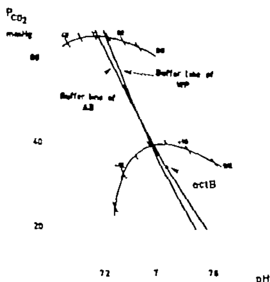


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TABLE II The results of the measurements in group II AB = arterial blood, WP = welling point blood

Rat	Sample	pH	P_{CO_2} mmHg	Actual HCO_3^- mM	Base excess mM	log P_{CO_2}/pH	Cross sector point P_{CO_2}/pH
1	AB 1	7 45	35 4	24 0	+0.7	−1 41	36.5,7.4[illegible]
	WP 1					−1.50	
2	AB 1	7 43	35 0	22.8	−0.5	−1.57	76 5 7.[illegible]
	WP 1					−[illegible].06	
3	AB 1	7 41	34.[illegible]	[illegible]1 7	−1 9	−1.52	46 0,7.370
	WP 1					−2.27	
4	AB 1	7 46	36.6	[illegible]5.7	+2.3	−1 48	38 0,7 459
	WP 1					−1 98	
	AB 2	7 49	29 7	22.2	+0.6	−1.59	46 3,7.367
	WP 2					−[illegible].00	
5	AB 1	7 47	3[illegible]3	[illegible]3[illegible]	+1[illegible]	−1 79	55 0,7 3[illegible]
	WP 1					−2.04	
	AB 2	7 46	34 0	23 8	+1 4	−1 73	50 7.3[illegible]
	WP					−2.10	

man was used to evaluate the acid-base parameters. Fig. 5 shows the buffer line of arterial blood and of blood from a welling point at one measurement The former line was obtained by the Astrup technique and the latter by the equilibration ultramicro technique. In all cases the absolute value of the slope (log P_{CO_2}/pH) of the buffer line for welling point blood was greater than that for the arterial blood ($P < 0.01$) as shown in Table II which means that the welling point blood had a higher buffer capacity In Fig. 6 the calculated mean values of the acid-base parameters of the welling point and arterial blood are represented as two buffer lines. The buffer line of the welling point blood is given as the mean slope (−1 96 log P

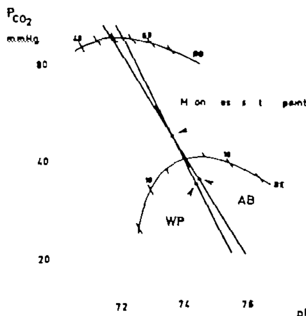


Fig 6. The mean buffer lines of the arterial (AB) and the welling point (WP) blood. The mean P_{CO_2} obtained for AB in group I + II and the mean P_{CO_2} for WP in group I are indicated, as well as the mean point of intersection of the buffer lines. For abbreviations and re-drawing of the diagram, see Fig 5

units) and the mean of the point of intersection between the two blood buffer lines from y single experiment (P_{CO_2}/pH = 48.4/7.357). The mean P_{CO_2} (±1 S.D.) of the arterial d in the two groups was 34.2 ± 1.1 mmHg and that of the welling point blood 33.3 ± 1.2 Hg (P_{CO_2} microelectrode recordings). The pH of the welling point blood obtained from nomogram (Fig. 6) was 7.437 thus slightly lower than the value of 7.448 of the arterial d.

$$pH = pK + \log[HCO_3^-] - \log S \cdot P_{CO_2} \quad (1)$$

actual bicarbonate concentration was calculated by using the Henderson-Hasselbalch ation (1), where pK the apparent dissociation constant (6.10) for blood and S = the bility coefficient of carbon dioxide (0.03 mM/mmHg) for blood (Siggaard Andersen 7). For the arterial and welling point blood the [HCO_3^-] was 22.9 and 21.7 mM respecly.

Discussion

P_{CO_2} microelectrode. The silicone plug in the tip of the electrode must adhere properly to ner surface of the tip so as to avoid leakage of water and electrolytes. Further the plug be fairly thin, otherwise the response time will be too long. The lifetime of the electrode fluenced by the bicarbonate sensitivity of the antimony electrode. This phenomenon has discussed by Karlmark and Sohtell (1973) and Puschett and Zurbach (1974). This interce, however does not affect the sensitivity or the validity of the electrode, as it is reedly calibrated for known CO_2 tensions.

he empirical temperature coefficient for the antimony electrode (Vieira and Malnic 1968) .3 mV/°C, which means about 0.05 log P_{CO_2}/°C. This figure means an error of about 5 Hg in the area 40 mmHg P_{CO_2} for each C°. The influence of temperature is thus substantial checking of the temperature of the tissue surface, as well as of the bottom of the calibracups, is therefore of great importance. This was done repeatedly by a thermistor with a meter of 1.0 mm.

he intratubular P_{CO_2}. The intratubular P_{CO_2} range obtained in this investigation (40–45 Hg) is in accordance with that reported by Karlmark and Danielson (1974) with use of equilibration technique. The values for intratubular P_{CO_2} obtained by direct measurements, supported the view that the P_{CO_2} of proximal tubular fluid is higher than that of the rial blood.

he high intraluminal P_{CO_2} was suggested by Brodsky and Schilb (1974) to be due to the acellular production of CO_2. However this suggestion is contradicted by the finding of wartz et al. (1971, 1974) that the P_{CO_2} of the epithelial cells of the turtle bladder in the ence of exogenous carbon dioxide was about 2 mmHg, and by the fact that Malnic and ebisch (1976) calculated the intracellular P_{CO_2} to be, at best, 2–6 mmHg above that of the ous blood.

The neutralization reaction between filtered HCO_3^- and secreted H^+ was proposed by ell (1978) to be located at the border of the tubular lumen and cell, and it is therefore ected that the high luminal P_{CO_2} probably emanates from this reaction. It is also sug-

gested that the reaction in itself should act as a limiting factor for CO diffusion out of lumen The P_{CO_2} of the luminal fluid has to be the built up to the same level as that of reaction centre. Not until this level is reached will there be a gradient for CO_2 transport out of the lumen.

The chemical equilibrium—non-equilibrium situation in the proximal tubule Rector et al (1965) and Vieira and Malnic (1968) found that the in situ pH of the tubular fluid is lower than the pH of a sample taken from the same tubular site and equilibrated to a P of 40 mmHg. The authors proposed that the difference between these two pH was due to chemical non-equilibrium of the carbonic acid-bicarbonate system. The high P_{CO_2} in the proximal tubule found in the present study shows that the collected sample should not be equilibrated with a P_{CO_2} of the blood level or a P_{CO_2} of 40 mmHg but with that of the luminal fluid The finding of a high intratubular P_{CO_2} thus seems to indicate that a chemical equilibrium exists between hydrogen ions, bicarbonate ions and carbon dioxide in the proximal tubular fluid It is therefore probable that the Henderson-Hasselbalch equation (I) is u... in calculating one of the variables if the other two are known

Welling point versus arterial blood The difference between the mean P_{CO_2} of the arterial blood in groups I and II combined and that of the welling point blood was 0.9 mmHg which is not statistically different from zero It was shown, however in the paired measurements in group I that the P_{CO_2} of the welling point blood was slightly lower than that of arterial blood ($P < 0.01$)

In the ultrafiltration process the efferent arteriolar blood will have a higher concentration of hemoglobin and plasma proteins as compared with the arterial blood. The buffer capacity will be increased due to the filtration process, as is shown by the higher absolute value log P_{CO_2}/pH for welling point than for arterial blood This is also illustrated by the fact that the increase in buffer base (BB) amounted to about 3 mM (Fig. 6) while the bicarbonate concentration was calculated to have decreased by 1.2 mM The small increase in buffer capacity might explain the small change in pH and the small but statistically significant decrease of P_{CO_2}

The rising protein concentration along the glomerular capillaries also means an increasing importance of the Donnan phenomenon, which thus slightly increases the load of bicarbonate to the proximal tubules.

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aline medium (pH 8) carbon dioxide rather would react with hydroxyl ions and the appears to have a site of OH^- or HCO_3^- and not for CO_2 or H_2CO_3 (Maren 1967) high entirely intracellular pH is, however improbable, but could very well exist on brane surface (Craxford et al 1938). This means that the OH^- ion, which is available redox splitting of water leaves the H^+ ion for secretion and reacts with carbon at the enzyme.

neutralization reaction between H^+ and HCO_3^- ions and the dehydration of H_2CO_3 een extensively investigated with micropuncture techniques since it has been possible form pH measurements in the tubules in vivo With glass microelectrodes Rector 1965) compared the in situ pH with the pH measured in a sample taken from the same and equilibrated to a P_{CO_2} of 40 mmHg. Under control conditions they found no nce in this respect in the proximal tubules After inhibiting the carbonic anhydrase etazolamide, the in situ pH was 0.85 pH units lower than the pH in the equilibrated e. This difference was thought to be due to the inhibition of the catalyzed dehydration bonic acid in the tubular lumen. This would lead to an accumulation of the carbonic nd thus also of free hydrogen ions. The authors concluded that an enzyme localized tubular luminal membrane would prevent this accumulation. By inhibiting carbonic drase ith acetazolamide and using antimony pH microelectrodes Vieira and Malnic) basically confirmed these results. Their pH difference was only 0.41 pH units, how- Both groups considered the pH difference to be due to a chemical nonequilibrium.

rkmark and Danielson (1974) measured the proximal intratubular pH in rats under al conditions and combined the results with the buffer lines obtained with a CO bration method (Karlmark and Sohtell 1973) Assuming a luminal chemical equi- m they were then able to calculate the intratubular P_{CO_2} The urine P_{CO_2} was found higher than the P_{CO_2} in the arterial blood. Recently Sohtell and Karlmark (1976) Sohtell (1978 b) measured the intratubular P_{CO_2} with a microelectrode and found that fers from the blood P_{CO_2} and that an intraluminal chemical equilibrium exists.

e aim of this investigation was to determine the pH bicarbonate concentration and along the rat proximal tubule under control conditions and during administration of zolamide in order to cast light on the problems concerning the equilibrium non- brium situation for pH and P_{CO_2} in the proximal tubular lumen.

Material and Methods

he rats (Sprague-Dawley strain, Anticimex, Stockholm, Sweden) weighing 205–285 g, fed on diet of rcial rat pellets (Anticimex, Sweden) were used. They were allowed food and water ad libitum until art of the experiment. The rats were anesthetized by an intraperitoneal injection of Inactin (Chem. Promonta, GmbH, Hamburg, W Germany) in dose of 120 mg/kg body weight (b.w.). body temperature was kept at 37.5°C ith servo-controlled heating table The rats were tracheoto- d and catheter was inserted into the left femoral vein for infusions The left femoral artery was eterized for blood sampling and the right femoral artery for blood pressure measurements. The urinary der was drained to avoid diminished urine flow (Karlmark and Danielson 1974). The left kidney was ded through flank incision and immobilized in lucite cup by embedding in 2% agar-in-Ringer tion. The exposed dorsal surface was superfused with warmed paraffin oil, preequilibrated with 5% on dioxide and 20% oxygen in nitrogen. The left ureter was catheterized. Inulin TF P inulin as measured, with FITC-inulin as test substance. A continuous infusion of

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CO_2 along the proximal tubules in the rat kidney

By

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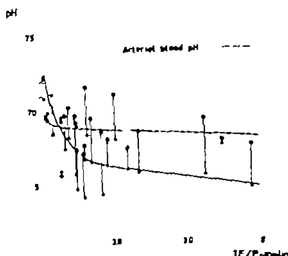
Abstract

SOHTELL, M CO_2 *along the proximal tubules in the rat kidney* Acta physiol. scand. *105* 146–155

The proximal intratubular pH of the rat kidney was measured in vivo with an antimony electrode ... P_{CO_2} and bicarbonate concentration of the proximal tubular fluid were determined with a ... equilibration system The tubular fluid to plasma inulin concentration ratio was evaluated by a micro fluorometric method The acid-base parameters and the inulin concentrations were determined ... control conditions and during acetazolamide treatment The intratubular P_{CO_2} was higher than the ... the systemic arterial blood under control conditions and the difference in P_{CO_2} was increased during ... zolamide treatment. In acetazolamide treated rats the rate of fractional bicarbonate reabsorption ... decreased in the early part of the proximal tubule while it was of about the same in the middle and late ... as compared with control rats. The total bicarbonate reabsorption in the proximal tubule was reduced ... 50% due to the carbonic anhydrase inhibition. It seems possible that the bicarbonate is still reabsorbed ... CO_2 after carbonic anhydrase inhibition, as hydrogen ion secretion is not totally stopped by this ... The increase in intratubular P_{CO_2} after acetazolamide treatment is assumed to be due to an inhibition ... carbonic anhydrase facilitating effect on outward diffusion of CO_2 from the tubular lumen across the ... wall

The urine in mammals may become acid either by reabsorbing filtered buffer anions, ... HCO_3^- and HPO_4^{2-} or by the addition of acid from the tubular cells to the fluid. It b... clear from the findings of Pitts and Alexander (1945) that a tubular hydrogen ion sec... had to exist. The secreted hydrogen ion will then react with the filtered bicarbonate ... forming carbonic acid which is decomposed into water and carbon dioxide. Rector (... Malnic and Steinmetz (1976) and Malnic and Giebisch (1976) have proposed that the ... portion of the bicarbonate reabsorption is mediated by the hydrogen-ion secretion. ... (1969) however has suggested a direct HCO_3 reabsorbing mechanism which in th... should be responsible for about 60% of the total reabsorption

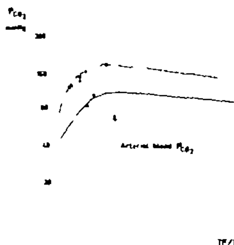
One intracellular source of the secreted hydrogen ions is generally considered to ... carbonic acid formed by the hydration of carbon dioxide. This reaction is catalyz... carbonic anhydrase, which enzyme is present in the rat tubular cells, both in the cyto... and in the cell membranes (Lönnerholm 1971 and Wistrand and Kinne 1977) The ... ions might also emanate from the splitting of water into hydrogen and hydro...



The intratubular pH under control conditions (filled circles and solid line) and during acetazolamide (open circles and dashed line), at the same puncture site, plotted against the functional length of ule under control conditions. The lines are drawn by eye. The dotted line indicates the mean arterial

Results

ean blood acid-base parameters did not differ significantly between the control period he period of acetazolamide treatment, according to paired t-test evaluation. Thus the values (±1 S.D.) of pH, HCO_3, P_{CO_2} and base excess for the control periods were ±0.02, 23.7±1.8 mM, 37.8±3.4 mmHg and 0.3±1.8 mM and during acetazolamide ion 7.41 0.03, 24.0 1.6 mM, 38.7±3.9 mmHg and −0.1±1.6 mM respectively



) The intratubular P_{CO_2} under control conditions (filled circles and solid line) and during acetazolamide ion (open circles and dashed line), at the same puncture site, plotted against TF/P-inulin under control ditions. The lines are drawn to the best visual fit. The dotted line indicates the mean arterial blood P_{CO_2}

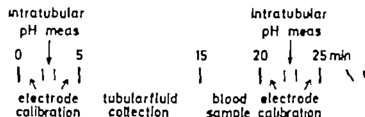


Fig. 1 The experimental arrangement.

3 0 ml/kg b.w and hour of 40 mg/ml FITC-inulin in Ringer solution was given. The inulin (Kabi, Spånga, Sweden) was labelled with fluorescein isothiocyanate (Pharmacia Fine Chemicals, Up Sweden). After 1 h the infusion gave a steady plasma concentration of about 1 mg/ml. In experime the carbonic anhydrase activity was inhibited the FITC-inulin Ringer solution was replaced by acetazolamide (Diamox® Lederle, Pearl River N Y), and 40 mg/ml FITC-inulin in 150 mM bicarbonate solution. The sodium bicarbonate was added to keep the acid-base status of the rat eq the two situations studied. The infusion was continued for 30 min before any sample was taken The mental arrangement is shown i Fig. 1

Intratubular pH The intratubular pH was measured with an a timony electrode system (Karlmark designed fo micropuncture, in a manner simila to that described by Vieira and Malnic (1968) The trodes were ground to a diameter of about 5 µm. The silver-silver chloride reference electrode had a junction of 1 M KCl (2 agar) in a glass cannula with a tip of 7 µm. The tip was inserted into the parenchyma and the location was not critical for the pH measurements. The electrodes ere connect mV meter (PHM 26, Radiometer Copenhagen, Denmark). They were calibrated before and the tratubular measurement, using phosphat buffer solutions placed in a holder adjacent to the kidn and thermostated to the kidney temperature

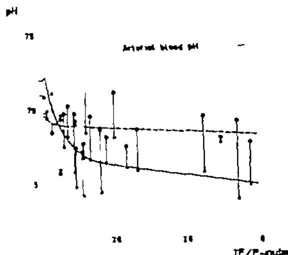
The sensitivity of the electrod system was of the same order as that described by Karlmark (19 Karlmark and Sohtell (1973) It was found necessary however to reinvestigate the bicarbonate interfe described by Karlmark and Sohtell (1973) because of the variation of the antimony quality and the ma electrode grinding. The terference was measured as described in the paper cited and was found to b pH units lower than the true pH value. All pH measurements containing bicarbonate recorded antimony electrode have therefore been corrected. Proximal tubules were identified as the non-fluore segments nd were randomly punctured.

Intratubular sampling After the pH measurements, tubula fluid was sampled at a rate of 3-4 µl using a collection method described by Karlmark and Danielson (1974). The puncture site was the sa the pH measurements and the tubular urine sampling. Dur g the period of acetazolamide infusion, the puncture site was reinvestigated for pH recording and sample collection. The results from the two situ could thus be compared at the same anatomical site. This point is expressed as TF/P inulin under or conditions.

Inulin determination. A fluorescence method described by Rutili *et al* (1976) was modified and w measure the FITC-inul n concentrations in plasma and urine (Sohtell *et al.* 1978). Duplicate samples of nl each of tubular fluid and arterial blood plasma were assayed for their FITC-inulin concentrations. TF/P ratio of the FITC-inulin indicated the distance between the glomerulus and the punctured site, be in mind that TF/P inulin does not linearly represent the length.

Intratubular P_{CO_2} and bicarbonate determinations. The intratubular fluid samples were equilibrated t ferent P_{CO_2} and the pH was measured at each gaseous tension by means of a glass microelectrode (Kar *et al.* 1971). From the measured intratubula pH and the pH/log P_{CO_2} line, the intratubular P_{CO_2} ca determined assuming conditions of equilibrium. The bicarbonate concentration of the sample at particular pH nd P_{CO_2} was then calculated using the Henderson-Hasselbalch equation. Aspects of determinations have been discussed by Karlmark and Sohtell (1973) and Sohtell and Karlmark (197

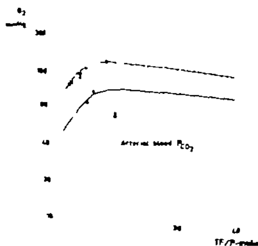
Acid-base of the blood. The acid-base status of the rat was determined as that of the arterial blo cording to the method of Siggaard Andersen (1967). The bicarbonate concentration was evaluated for pl water supposing the volume of plasma proteins is 6 of plasma volume (Kunau 1972). For the an 400 µl of blood was drawn from the left femoral artery into heparinized syringe without suction. 2 this volume was taken for determination of the plasma FITC-inulin concentration. After transient d the blood pressure should not be below 100 mmHg, otherwise the experiment was ended.



The intratubular pH under control conditions (filled circles and solid line) and during acetazolamide ...a (open circles and dashed line), at the same puncture site, plotted against the functional length of ...ule under control conditions. The lines are drawn by eye. The dotted line indicates the mean arterial

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TABLE I Results from 47 proximal tubules from 10 rats. C = control conditions and I = inhibition of carbonic anhydrase activity

Tubule	Intra tub pH	Intra tub. P_{CO_2} mmHg	Intra-tub. HCO_3^- mM	Intra tub. pH at blood P_{CO_2}	Non-equil. pH	TF/P Inulin	Blood pH	Blood P_{CO_2} mmHg	Plasma HCO_3^- mM	[illegible]
1c	6.84	36.0	6.1	6.82	+0.0	1.61	7.43	37.5	4.7	[illegible]
1I	7.16	53.0	18.7	7.34	−0.18	1.4	7.39	34.3	20.5	[illegible]
2c	6.39	115	6.9	7.39	−0.55	3.72	7.38	34.8	20.5	[illegible]
3c	6.44	—	—	—	—	1.81	7.38	34.8	20.5	[illegible]
4c	6.85	65.0	6.0	6.84	−0.26	1.24	7.38	34.8	20.5	[illegible]
2I	6.91	125	24.8	7.36	−0.45	1.79	7.35	42.5	23.8	[illegible]
3	6.86	121	21.4	7.31	−0.45	1.40	7.35	42.5	23.0	[illegible]
4I	6.57	243	22.0	7.28	−0.71	1.22	7.35	42.5	23.0	[illegible]
5c	6.61	128	12.7	7.07	−0.46	2.18	7.40	40.9	25.0	[illegible]
6c	6.42	159	10.2	6.94	−0.52	1.55	7.40	40.9	25.0	[illegible]
7c	6.59	80.0	7.6	6.85	−0.26	2.33	7.40	40.9	25.0	[illegible]
5I	6.75	202	27.7	7.39	−0.64	1.50	7.40	38.0	23.2	[illegible]
6I	6.71	227	28.4	7.39	−0.68	1.22	7.40	38.0	23.2	[illegible]
7I	6.86	148	26.1	7.36	−0.50	1.63	7.40	38.0	23.2	[illegible]
8c	6.48	132	9.7	6.87	−0.39	1.45	7.39	44.4	26.0	[illegible]
9c	6.67	82.5	9.4	6.90	−0.23	1.57	7.39	44.4	26.0	[illegible]
10c	6.64	90.0	9.6	6.88	−0.24	1.89	7.39	44.4	26.0	[illegible]
8I	6.74	197	26.4	7.[illegible]8	−0.54	1.34	7.37	46.0	25.6	[illegible]
9I	6.77	186	26.7	7.31	−0.54	1.25	7.37	46.0	25.6	[illegible]
10I	6.81	176	27.7	7.32	−0.51	1.50	7.37	46.0	25.6	[illegible]
11c	6.77	87.0	12.5	7.15	−0.38	3.49	7.43	36.5	23.9	[illegible]
12c	6.57	85.0	7.7	6.94	−0.37	3.26	7.43	36.5	23.9	[illegible]
11I	6.79	164	24.6	7.48	−0.69	1.00	7.44	36.8	[illegible]4.7	[illegible]
12I	6.94	137	[illegible]9.1	7.48	−0.54	—	7.44	36.8	24.7	[illegible]
13c	6.49	101	7.6	6.92	−0.43	3.90	7.40	38.2	23.3	[illegible]
14c	6.66	69.0	7.7	6.92	−0.26	1.67	7.40	38.2	23.3	[illegible]
15c	6.64	100	10.6	7.07	−0.43	1.67	7.40	38.2	23.3	[illegible]
13I	6.77	179	25.7	7.44	−0.67	1.67	7.40	41.8	25.4	[illegible]
14I	6.95	119	25.8	7.43	−0.48	—	7.40	41.8	25.4	[illegible]
16c	6.99	69.0	16.4	7.26	−0.27	1.06	7.44	37.6	[illegible]5.2	[illegible]
17c	6.75	72.0	9.9	7.02	−0.27	1.30	7.44	37.6	25.2	[illegible]
16I	6.94	112	23.8	7.35	−0.41	1.06	7.4	40.2	25.8	[illegible]
17I	6.97	119	27.1	7.41	−0.44	1.15	7.42	40.2	25.8	[illegible]
18c	6.58	56.0	5.2	6.78	−0.20	1.45	7.44	31.6	21.5	[illegible]
19c	6.81	68.5	10.8	7.10	−0.29	2.01	7.44	31.6	21.5	[illegible]
20c	6.96	37.5	8.3	7.02	−0.06	1.26	7.44	31.6	21.5	[illegible]
18	6.97	86.0	19.6	7.34	−0.37	1.13	7.45	35.0	24.5	[illegible]
19I	7.11	64.0	[illegible]0.1	7.34	−0.23	1.[illegible]6	7.45	35.0	24.5	[illegible]
20I	6.93	115	23.9	7.40	−0.47	1.13	7.45	35.0	24.5	[illegible]
21c	7.09	63.5	19.0	7.31	−0.22	0.96	7.39	37.8	22.5	[illegible]
21I	6.96	107	23.8	7.54	−0.58	1.09	7.45	34.0	23.5	[illegible]
22c	6.82	67.5	10.9	7.06	−0.24	1.35	7.42	38.2	24.5	[illegible]
23c	6.90	49.0	9.5	7.01	−0.11	1.45	7.42	38.2	24.5	[illegible]
24c	7.11	57.0	17.9	7.28	−0.17	1.13	7.42	38.2	24.5	[illegible]
22I	7.02	111	28.3	7.47	−0.45	1.13	7.40	38.7	23.6	[illegible]
23I	6.92	139	28.2	7.44	−0.5[illegible]	1.22	7.40	38.7	23.6	[illegible]
24I	6.84	168	28.3	7.45	−0.61	1.08	7.40	38.7	23.6	[illegible]

TF/P$_{HCO_3}$ 2.0

1 2 3 TF/P-inulin

The tubular fluid–plasma [concen]tration ratio of bicarbonate [under] control conditions (filled [circles]) and during acetazolamide [administratio]n (open circles), in relation [to T]F/P inulin under control [conditi]ons

[Int]ratubular pH. As discussed in a previous paper (Sohtell 1977) a touch on the luminal [m]embrane with a measuring electrode would show a potential recording giving a false [v]alue. By moving the electrode from the cell border such a potential effect was avoided. [In Fi]g. 2 the intratubular pH measured during the control and acetazolamide periods is [show]n, in relation to the functional length of the tubule under control conditions (TF/P [inulin]).

[Intr]atubular P_{CO_2} values were obtained from the pH/log P_{CO_2} line and the corresponding [intra]tubular pH, as previously described. In Fig. 3 the intratubular P_{CO_2} under control and [acet]azolamide conditions is shown in relation to TF/P-inulin in the control situation. It is [seen] that the P_{CO_2} increased rapidly in the early parts of the proximal tubule, with a tendency [to d]ecline in the later parts. Most values were higher than the P_{CO_2} of the arterial blood [(P<]0.001). Further the P_{CO_2} values obtained during acetazolamide administration were [high]er than those in the control situation ($P<0.001$).

[Th]e intratubular bicarbonate concentration. In the calculations of bicarbonate the constants [use]d in the Henderson-Hasselbalch equation were $pK_a = 6.101$ and $S = 0.0307$ which are [foun]d for the ultrafiltrate of the rat plasma (Sohtell and Karlmark 1978). These values are, [fur]thermore, in agreement with those of human plasma filtrate (Siesjö 1962, Mitchell *et al.* [196]5 and Sohtell 1971) and with those calculated as described by Hastings and Sendroy [(19]25) and Van Slyke *et al.* (1928). The bicarbonate concentrations are seen in Table I, [and i]n Fig. 4 the tubular fluid-plasma ratio (TF/P) for bicarbonate under control and [acet]azolamide conditions is shown as a function of TF/P inulin in the control situation.

Discussion

[Ka]rlmark and Sohtell (1973), Malnic *et al.* (1974) and Puschett and Zurbach (1974) have [rep]orted that falsely low pH values are recorded with the antimony electrode when it is [use]d for measurements in solutions containing bicarbonate and is standardized in phosphate [buf]fers. The differences ranged between 0.07 and 0.18 pH units. If the properties of the [elec]trodes are changed, and with somewhat different standardizations, however, a re-evalua[tion] of the magnitude of the error is required for every experimental set-up. In this investiga-

TABLE I. Results from 47 pro[illegible]imal tubules from 10 rats. C = control conditions and I [illegible] anhydrase activity

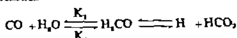
Tubule	Intra tub pH	Intra tub P_{CO_2} mmHg	Intra tub. HCO^- mM	Intra tub. pH at blood P_{CO_2}	Non-equil. pH	TF/P inulin	Blood pH	Blood P_{CO_2} mmHg	Plasma HCO_3^- mM	[illegible]
1c	6.84	36.0	6.1	6.82	+0.0[illegible]	1.61	7.43	37.5	24.7	[illegible]
1i	7.16	53.0	18.7	7.34	−0.18	1.42	7.39	34.3	20.5	[illegible]
2c	6.39	115	6.9	7.39	−0.55	3.72	7.38	34.8	20.5	[illegible]
3c	6.44	—	—	—	—	1.81	7.38	34.8	20.5	[illegible]
4c	6.85	65.0	6.0	6.84	−0.26	1.24	7.38	34.8	20.5	[illegible]
2i	6.91	125	24.8	7.36	−0.45	1.79	7.35	42.5	23.0	[illegible]
3i	6.86	121	21.4	7.31	−0.45	1.40	7.35	4[illegible].5	23.0	[illegible]
4i	6.57	43	[illegible]2.0	7.28	−0.71	1.22	7.35	4[illegible].5	23.0	[illegible]
5c	6.61	128	12.7	7.07	−0.46	2.18	7.40	40.9	25.0	[illegible]
6c	6.42	159	10.2	6.94	−0.5	1.55	7.40	40.9	25.0	[illegible]
7c	6.59	80.0	7.6	6.85	−0.26	2.33	7.40	40.9	25.0	[illegible]
5i	6.75	202	27.7	7.39	−0.64	1.50	7.40	38.0	23.2	[illegible]
6i	6.71	227	28.4	7.39	−0.68	1.2[illegible]	7.40	38.0	23.2	[illegible]
7i	6.86	148	[illegible]6.1	7.36	−0.50	1.63	7.40	38.0	23.2	[illegible]
8c	6.48	132	9.7	6.87	−0.39	1.45	7.39	44.4	26.0	[illegible]
9c	6.67	82.5	9.4	6.90	−0.23	1.57	7.39	44.4	26.0	[illegible]
10c	6.64	90.0	9.6	6.88	−0.24	1.89	7.39	44.4	26.0	[illegible]
8i	6.74	197	26.4	7.28	−0.54	1.34	7.37	46.0	25.6	[illegible]
9i	6.77	186	26.7	7.31	−0.54	1.25	7.37	46.0	[illegible]5.6	[illegible]
10i	6.81	176	27.7	7.3	−0.51	1.50	7.37	46.0	25.6	[illegible]
11c	6.77	87.0	1[illegible].5	7.15	−0.38	3.49	7.43	36.5	23.9	[illegible]
12c	6.57	85.0	7.7	6.94	−0.37	3.26	7.43	36.5	23.9	[illegible]
11i	6.79	164	24.6	7.48	−0.69	1.00	7.44	36.8	24.7	[illegible]
12i	6.94	137	29.1	7.48	−0.54	—	7.44	36.8	24.7	[illegible]
13c	6.49	101	7.6	6.92	−0.43	3.90	7.40	38.2	23.3	[illegible]
14c	6.66	69.0	7.7	6.9	−0.[illegible]6	1.67	7.40	38.2	23.3	[illegible]
15c	6.64	100	10.6	7.07	−0.43	1.67	7.40	38.2	[illegible]3.3	[illegible]
13i	6.77	179	25.7	7.44	−0.67	1.67	7.40	41.8	25.4	[illegible]
14i	6.95	119	[illegible]5.8	7.43	−0.48	—	7.40	41.8	[illegible]5.4	[illegible]
16c	6.99	69.0	16.4	7.26	−0.27	1.06	7.44	37.6	25.2	[illegible]
17c	6.75	72.0	9.9	7.02	−0.27	1.30	7.44	37.6	[illegible]	[illegible]
16i	6.94	112	[illegible]3.8	7.35	−0.41	1.06	7.4	40.2	25.8	[illegible]
17i	6.97	119	27.1	7.41	−0.44	1.15	7.42	40.2	[illegible]5.8	[illegible]
18c	6.58	56.0	5.2	6.78	−0.20	1.45	7.44	31.6	1.5	[illegible]
19c	6.81	68.5	10.8	7.10	−0.29	2.01	7.44	31.6	1.5	[illegible]
20c	6.96	37.5	8.3	7.02	−0.06	1.26	7.44	31.6	21.5	[illegible]
18i	6.97	86.0	19.6	7.34	−0.37	1.13	7.45	35.0	4.5	[illegible]
19i	7.11	64.0	20.1	7.34	−0.23	1.26	7.45	35.0	[illegible]4.5	[illegible]
20i	6.93	115	23.9	7.40	−0.47	1.13	7.45	35.0	4.5	[illegible]
21c	7.09	63.5	19.0	7.31	−0.22	0.96	7.39	37.8	22.5	[illegible]
11	6.96	107	23.8	7.54	−0.58	1.09	7.45	34.0	23.5	[illegible]
22c	6.82	67.5	10.9	7.06	−0.24	1.35	7.42	38.2	24.5	[illegible]
23c	6.90	49.0	9.5	7.01	−0.11	1.45	7.42	38	24.5	[illegible]
24c	7.11	57.0	17.9	7.28	−0.17	1.13	7.42	38.2	4.5	[illegible]
22i	7.02	111	28.3	7.47	−0.45	1.13	7.40	38.7	23.6	[illegible]
23i	6.92	139	28.2	7.44	−0.5	1.22	7.40	38.7	23.6	[illegible]
4i	6.84	168	[illegible]8.3	7.45	−0.61	1.08	7.40	38.7	23.6	[illegible]

hazardous, as pH and P_{CO_2} are not constant along the tubules, which means that the tions are greatly dependent on the puncture site from which the recordings and samples ate.

...shky and Schäfb (1974) suggested that an elevation of the P_{CO_2} of the lumen might ...e to an intracellular carbon dioxide production. Malnic and Giebisch (1976) have, ...ver calculated the intracellular P_{CO_2} to be, at best, 2–6 mmHg above that of the venous ...l and/or the tubular fluid. Schwartz *et al.* (1971 1974) have also calculated the P_{CO_2} ...thelial cells of the turtle bladder to be about 2 mmHg in the absence of exogenous ...on dioxide. It is therefore unlikely that the high intratubular P_{CO_2} reflects a high intra ...llar P_{CO_2}.

...w investigations have been made concerning the carbon dioxide permeation across ...gical membranes. Constantine *et al* (1962) measured the half time for carbon dioxide ...ke by erythrocytes and found it to be about 0.1 second, and the same value was found ...rew *et al* (1963) in similar experiments.

...om the chemical reaction



$$CO_2 + H_2O \underset{K_2}{\overset{K_1}{\rightleftharpoons}} H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

...and Maren (1972) determined the rate constants K_1 and K_2 at 37°C and found a half ...of 0.014 second for K_1 (uncatalyzed). This reaction half time indicated that the trans... of CO_2 through erythrocyte membranes rather is restricted in CO_2 diffusion than de...lent on the uncatalyzed dehydration reaction.

...experiments designed to evaluate the acidification kinetics in single nephron segments, ...tte *et al.* (1974) provided some evidence of restriction of CO_2 diffusion through the ...ximal tubular wall. A half time of 0.5 to 1.0 s was found for the carbon dioxide equi...ation in air-equilibrated solution perfusing the proximal tubules, and this was much ...er than the half time of getting an equilibrium in the CO_2 hydration-H_2CO_3 dehydration ...tion, even without carbonic anhydrase. They suggested that the equilibration rate is ...ted by the diffusion of carbon dioxide across the tubular wall.

...ohtell (1978 b) demonstrated by direct intratubular measurements that the P_{CO_2} in the ...ximal tubules varied between 40 and 85 mmHg in rats under control conditions. This ...tly strengthens the result of the present investigation. The similarity of this range for ...control situation in the present study to that found by Karlmark and Danielson (1974) ...ngly supports the existence of chemical equilibrium in the reactions within the carbonic ... system contained in the tubular fluid. This qualifies the equilibration method (Karl...rk and Sohtell 1973) for use in calculating the intratubular P_{CO_2}. The high intratubular ...as compared with the blood P_{CO_2}, points to a restriction of the CO_2 diffusion out of ...lumen.

...t was proposed by Sohtell (1978 a), from investigations of the neutralization reaction ...ween H^+ and HCO_3^- ions in a multi-membrane model, that the reaction in itself causes ...ntation of the CO_2 diffusion if the reaction centre is located close to the membrane of ...luminal side. This means that the luminal P_{CO_2} has to be increased to a level equal to the ...P_{CO_2} level in the reaction centre, if a net outward transport of carbon dioxide is to take place.

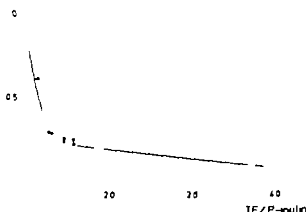


Fig. 5. The fractional bicarbonate excretion under control conditions (filled circles and solid line) and during inhibition of carbonic anhydrase activity (open circles and dashed line), plotted against TF/P-inulin under control conditions. The lines are drawn b

tion pH values obtained with the antimony electrode were corrected for an error of 0.0 units (see Material and Methods)

Direct measurements of the pH within the rat proximal tubules under free-flow conditions in normal situations and during carbonic anhydrase inhibition have been reported in the literature (see Rector 1976) The pH patterns along the proximal tubules (Fig. 2) in the present investigations, under control conditions are consistent with the results obtained earlier A remarkable change of pH is seen when acetazolamide is given. In the early proximal tubule (TF/P inulin < 1.26) the pH is lower than under control conditions, in the later parts it is higher Quehenberger *et al* (1977) reported similar results, but a transient pH decrease in the early proximal tubules just after benzolamide infusion functional background of the change of the pH pattern after administration of acetazolamide is not fully understood.

Under control conditions the bicarbonate reabsorption expressed as fractional bicarbonate excretion, in the early part of the proximal tubule was much faster than in the late (Fig. 5) After the administration of acetazolamide the reabsorption was almost the same along the whole tubule and of about the same rate as in the late tubule under control conditions. These discrepancies under the two conditions might explain the results presented in Fig. 2. An altered pH pattern would also be the result of CO diffusion across the tubular membrane was restricted and H secretion into the tubular lumen decreased on administration of acetazolamide If so the restricted CO diffusion might be explained by the increased intratubular P_{CO_2} after acetazolamide treatment, as shown in Fig. 3

The difference between the in situ pH and the "equilibrated sample" pH obtained in control situation is identical with that calculated from the data of Karlmark and Danielson (1974), namely 0 29 which is statistically different from zero ($P < 0.001$). Rector *et al* (1965) and Vieira and Malnic (1968) found no such difference, except during carbonic anhydrase inhibition. Rector *et al* then found a value of 0.85 (benzolamide) and Vieira and Malnic 0.41 (acetazolamide), which may be compared with the 0.51 pH units found in this investigation (acetazolamide) Comparison of the mean pH-difference values is

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Berliner (1957) Walser and Mudge (1960) and Rector *et al.* (1960) suggested the e of a luminally active carbonic anhydrase. Rector *et al* (1965) proposed that this enzy creased the dehydration rate of H_2CO_3, preventing the accumulation of carbonic acid i tubular lumen and, thus, reducing the chemical non-equilibrium pH However the of Malnic *et al* (1974) pointed to the carbon dioxide diffusion as being the rate-li step rather than the dehydration reaction Maren (1967) questioned the necessity of a lu carbonic anhydrase for the dehydration of H_2CO_3.

The membrane bound enzyme found by Wistrand and Kinne (1977) would rather to facilitate the diffusion of carbon dioxide from the tubular lumen. This conclusion to be plausible, as it is shown in the present investigation that the intraluminal P significantly increased when the carbonic anhydrase is inhibited with acetazolamide. T view is also favoured by results of Karlmark *et al* (1977), who found no significant e on the in situ pH measured in perfused segments of the proximal tubules when using carbonic anhydrase inhibitors acting only in the tubular lumen

The results of this investigation indicate the existence of a high P_{CO_2} in the pro tubules, which might explain the earlier assumption of a non-equilibrium pH. The phe non is thus instead a non-equilibrium P_{CO_2}.

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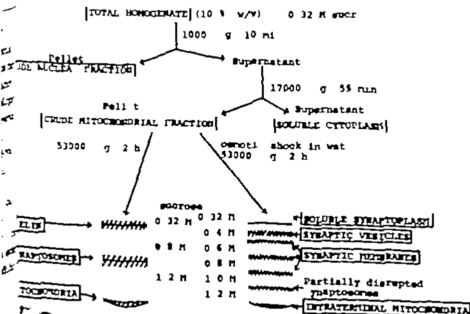
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[illegible] I Properties of amino acids injected intracerebrally into mice in the present study

acid	Specific activity	Amount per animal	Radioactivity per animal	Biological nature of amino acid
[illegible]	550 Ci/mol	56 nmoles	20 μCi	Slowly-metabolizing, transmitter candidate
[illegible]leucine	300 Ci/mol	2 nmoles	250 nCi	Non-metabolizing, non-transmitter
[illegible]	18 Ci/mmol	40 pmoles	1 μCi	Metabolizing, non-transmitter
[illegible] acid	275 Ci/mol	1.25 nmoles	500 nCi	Metabolizing, transmitter candidate

[illegible] was opened by drilling hole 2 mm from the intersection of the sagittal and coronal sutures [illegible] and McCormick 1957 Noble *et al.* 1967). One μl of the radioactive amino acids was injected through [illegible] syringe to depth of 2 mm into the cerebri. After removal of the needle the hole in the skull [illegible] covered with Pabrin-55 resin. A total of 20 mice was treated similarly in each series of expts., and the [illegible] was repeated four times with each amino acid. The properties and nature of the amino acids are [illegible] Table 1. All were obtained from the Radiochemical Centre, Amersham.

[illegible] mice were fatigued and cataleptic for 4–5 h after the injection, but gradual normalization of their [illegible] activities after 5 h was accompanied by progressive recovery of their normal alertness. The mice [illegible] then killed by decapitation 20 h after the injection, and the cerebri removed, pooled, weighed and [illegible] homogenized in cold 0.32 M sucrose. The homogenates were fractionated according to the [illegible] of Whittaker and Barker (1972) and samples taken from each fraction for radioactivity and protein [illegible] (see Fig. 1). Protein was determined by the method of Lowry *et al.* (1951). For the measure-



[illegible] for the collection of subcellular fractions of mouse brain for protein and radioactivity [illegible] This scheme is that of Whittaker and Barker (1972) with some modifications in the names

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From the Department of Biochemistry University of Oulu, Finland

Synaptosomal accumulation of intracerebrally injected amino

By

ANNELI KARPPINEN ELSA KUMPULAINEN and P LÄHDESMÄKI

Received 4 May 1978

Abstract

KARPPINEN, A., E. KUMPULAINEN and P LÄHDESMÄKI *Synaptosomal accumulation of intracerebrally injected amino acids* Acta physiol. scand 1979 *105* 156–162.

The distribution of the labels of [^{35}S]taurine, [^{3}H]lysine [^{14}C]glutamate and [^{14}C]norleucine in mouse brain subcellular fractions was followed after intracerebral injection *in vivo* [^{35}S]taurine [^{3}H]lysine and [^{14}C]glutamate and its metabolites accumulated in the nerve terminals, lysine and glutamate also occurring in the synaptic vesicles, while taurine remained mainly in the soluble synaptoplasm. [^{14}C]norleucine penetrated the brain cell membranes slowly but was still bound to the synaptic vesicles to a greater extent than ...

Key words. intracerebral injection, accumulation of amino acids, nerve terminals, synaptic organelles

In agreement with the work of De Belleroche and Bradford (1973), we have observed the presence of 10 amino acids in calf brain synaptic vesicles (Lähdesmäki and Winter 19..., Lähdesmäki *et al* 1977 a), although some other workers assume that their observations of the occurrence of these same amino acids in the synaptic vesicle fraction are influenced by contamination from amino acids in the soluble synaptoplasm (Mangan and Whittaker 1966, Rassin 1972) One further approach to the study of this controversial question would be to follow the passage of injected amino acids into brain subcellular fractions (Rassin *et al* 1977) This is complicated however by the effective blood brain barrier which prevents the passage of amino acids from the circulation into brain cells (Cohen and Lajtha 1972) Thus intracerebral injection may provide a better opportunity for explaining the entrance of amino acids into brain cells. Farrow and O Brien (1971) did in fact observe intraventricularly injected [^{3}H]acetate and amino acids formed from it in synaptosomes and synaptic vesicles. The work reported here examines the distribution of the radioactivity of four different amino acids in mouse brain subcellular fractions in order to ascertain whether these are able to accumulate in nerve terminals and their subcellular organelles

Material and methods

A total of 360 adult female white mice (NMRI) were used. They were anesthetized by an intramuscular injection of 200 µl of 10% (w/v) chloral hydrate and immobilized on a small animal rack with the head in horizontal position. A midsagittal incision was made from the eyes to the ears and the ... skull

II Recovery rates of the intracerebrally injected amino acids.

acid	Recovery %
aurine	21.4±2.2
ysine	20.7±1.8
lutamic acid	16.2±2.0
orleucine	14.8±2.4

ercentage recovery rates are calculated from the counts obtained in the total homogenates as com- with the radioactivity injected into the mouse brain.

ted, the counts in the total homogenates of the cerebri were taken as 100% and those her fractions expressed relative to these.

lysine penetrated the subcellular particles most rapidly and [^{14}C]norleucine most y [^{35}S]taurine also accumulated in the synaptosomes, but remained in the soluble toplasm and penetrated the synaptic vesicles and intraterminal mitochondria extremely y. Only a very small portion of the [^{35}S]taurine was attached to the myelin and synaptic ranes. [^{14}C]glutamate reached all the subcellular fractions, but was present in the hondria in relatively low amounts. [^{14}C]norleucine occurred in all the fractions, but in very minute quantities except in the synaptosomal fractions, where it occurred in r amounts than taurine. The percentage recovery rates for the amino acids, as calculated the label in the total homogenates of the cerebri, were about 21% for [^{35}S]taurine and ysine, but significantly less for [^{14}C]glutamate and [^{14}C]norleucine (Table II).

Discussion

ough there are several possible sources of error associated with a study like that re- ted here, the experiments nevertheless clearly demonstrate penetration of various sub- lar fractions by labelled amino acids when injected intracerebrally. The difficulties en- tered in the intracerebral injection of amino acids may be caused by the different types istribution possible in brain matter. One portion will certainly fall into the blood capil- es and be rapidly eliminated through the circulation, another portion may reach the in ventricles and be diluted by distribution into the brain-cerebrospinal fluid, one por n may perhaps fall into the extracellular space, and another directly into the neurons and al cells. This may be compensated for, however, by the use of several animals in one eriment.

elatively clear and interesting differences in distribution could be observed between the erent amino acids. The four amino acids used are of different metabolic and neuro- siological types. 1) taurine, for which a neurotransmitter or modulator role has been posed (Kaczmarek and Davison 1972, Oja and Lähdesmäki 1974), but which is meta- ically very stable (Lähdesmäki and Korhonen 1978), 2) lysine, which metabolizes slowly t hich is known to occur in synaptic vesicles (Lähdesmäki and Winter 1977), 3) glutamic d, which is apparently a neurotransmitter with effective transport systems into the aptosomes (Lähdesmäki et al. 1975) and synaptic vesicles (Lähdesmäki et al. 1977 a)

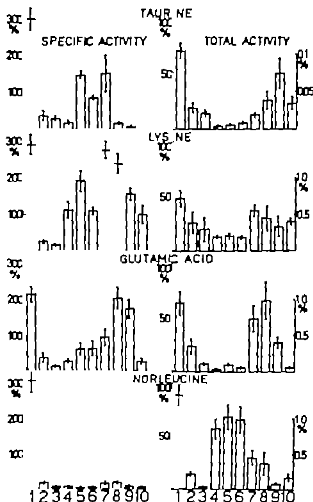


Fig. 2. Distribution of radioactivity ... intracerebral injection of [35S]taurine, [3H]ly sine [14C]glutamate and [14C]norleu mouse brain subcellular fractions in the ... of specific activity (counts min^{-1} g^{-1} pro tein) and total activity (counts min^{-1} g^{-1} original wet weight). The counts obtained ... the total homogenates of the cerebri for each amino acid were taken as 100 and the ... the other fractions expressed relative to it. 1 – soluble cytoplasm, 2 – crude nuclear fr tion, 3 – crude mitochondrial fraction, ... myelin, 5 – synaptosomes, 6 mitochondria, 7 – soluble synaptoplasm, 8 synaptic ... sicles, 9 – synaptic membranes, 10 ... terminal mitochondria. Results (mean ... S.D indicated by vertical bars) are for ... experiments with 15-20 animals in each

ment of radioactivity each fraction was dissolved in 0.1 M NaOH mixed in scintillation liquid of Triton X 100 and 60% toluene containing 6 g/l 2,5-diphenyloxazol and 150 mg/l p-bis[2-(5-phenyl-oxazolyl)]benzene and then counted on Wallac liquid scintillation spectrometer (ULTROBETA 1210) ... described previously (Lähdesmäki et al. 1977 b).

In order to eliminate the effect of the label distributed in the subcellular fractions in consequence of homogenization, the following control experiment was also performed. The cerebri of untreated mice were removed and the corresponding amou t of labelled amino acids injected in the earlier experiments added to the homogenizing medium and homogenizatio and fractionation performed as above. The label distributed I the subcellular fractions in these latter experiments was then subtracted from that observed in the earlier experiments to btain real counts fo the radioactivity which had penetrated the cell membranes.

Results

The distribution of the radioactivity of the amino acids among the subcellular fractions indicated in Fig. 1 is depicted in Fig. 2 in the form of specific activity (counts min^{-1} g^{-1} protein) and total activity (counts min g^{-} original wet weight), the counts for the control expts. having first been subtracted from the values obtained In order to eliminate the effect of any variation between the different amino acids in the amounts of radioactivity

1 Recovery rates of the intracerebrally injected amino acids.

acid	Recovery %
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[illegible] recovery rates are calculated from the counts obtained in the total homogenate [illegible] with the radioactivity injected into the mouse brain.

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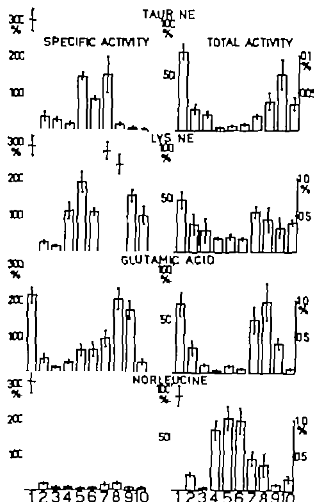


Fig. 2. Distribution of radioactivity after intracerebral injection of [³⁵S]taurine, [¹⁴C]lysine, [¹⁴C]glutamate and [¹⁴C]norleucine in mouse brain subcellular fractions in terms of specific activity (counts min⁻¹ g⁻¹ protein) and total activity (counts min⁻¹ g⁻¹ original wet weight). The counts obtained in the total homogenates of the cerebri for each amino acid were taken as 100 and the counts in the other fractions expressed relative to these. 1 = soluble cytoplasm, 2 = crude nuclear fraction, 3 = crude mitochondrial fraction, 4 = myelin, 5 = synaptosomes, 6 = mitochondria, 7 = soluble synaptoplasm, 8 = synaptic vesicles, 9 = synaptic membranes, 10 = nerve terminal mitochondria. Results (mean ± S.D., indicated by vertical bars) are from experiments with 15–20 animals in each.

ment of radioactivity each fraction was dissolved in 0.1 M NaOH, mixed in scintillation liquid (Triton X 100 and 60% toluene containing 6 g/l 2,5-diphenyloxazol and 150 mg/l p-bis[2-(5-phenyloxazolyl)]benzene and then counted on a Wallac liquid scintillation spectrometer (ULTROBETA 1210) as described previously (Lähdesmäki *et al.* 1977 b).

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) and also specific receptor sites at the synaptic membranes (Michaelis *et al.* 1974, [illegible]enmiki *et al.* 1977 b). There is strong evidence that glutamate is an excitatory neuro-[illegible]mitter in the central nervous system (Curtis and Johnston 1974).

[illegible]ucine

[illegible]ucine is a completely inert amino acid in the central nervous system both metabolically [illegible]eurochemically. It does not occur naturally but is not poisonous, for example, and [illegible]e used in comparative studies. It penetrated the brain cells and cell particles very slowly [illegible]mpared with other amino acids used, and its low recovery rate is indicative of relatively [illegible]ve excretion during the expt. The blood-brain barrier apparently allows an foreign [illegible]ance such as norleucine to penetrate easily in both directions.

[illegible] thus appears that intracerebrally injected amino acids, and principally those which [illegible]ally occur in brain tissue and subcellular particles and which apparently have a neuro-[illegible]iological action, may be exchanged between the intracerebral and intracellular amino pool. This in turn leads to their accumulation in nerve terminals, mitochondria and [illegible]ptic vesicles.

[illegible]tudy [illegible]as supported by the Research Foundation of the Orion Corporation and the National Research [illegible]cil for the Natural Sciences, Finland.

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[illegible], P. A. Karppinen, H. Saarni and R. Winter, Amino acids in the synaptic vesicle fraction [illegible] calf brain. Content, uptake and metabolism. *Brain Res.* 1977 a. 138 295–308.

and also having specific membrane receptors at the synaptic membranes (Lähdesmäki et 1977 b) but which is metabolically labile, and 4) norleucine, which is a completely compound in the central nervous system.

Taurine

Taurine penetrated the nerve terminals relatively rapidly but remained in the sol synaptoplasm and penetrated the synaptic vesicles and mitochondria and became attac at the synaptic membranes only very slowly Earlier studies show taurine to be one of most abundant amino acids in synaptic vesicles (Lähdesmäki and Oja 1976, Lähdesm *et al* 1977 a, Lähdesmäki and Winter 1977) and it also occurs in mitochondria (Lähd mäki and Korhonen 1978) Its exchange rate between the synaptoplasm and synaptoso particles seems to be very slow as it is also between the blood and brain (Oja *et al* 19 Similarly it has only 1/20 of the binding sites possessed by GABA at the synaptic membra (Kumpulainen *et al* 1978). Apparently all of [^{35}S]taurine injected remained in this form, d to its extremely slow metabolism in the central nervous system (Lähdesmäki and Korho 1978)

Lysine

[^{3}H]lysine penetrated the mitochondria, nerve terminals and synaptic vesicles most rap and was attached in the myelin and synaptic membranes in the largest quantities. Its change rate between the soluble cytoplasm and subcellular particles may be rapid, bu also has a high propensity to become bound at the membranes. This may be derived fr its basic nature The concentration of lysine in the synaptic vesicles is of the same order magnitude as that of glycine and alanine, for example (Lähdesmäki and Winter 19 Lysine can be incorporated into proteins, but otherwise its metabolism in the central nerv system is relatively slow (Strecker 1970) The similar recovery rates of lysine and tau in the total homogenates are indicative of their slow metabolism.

Glutamic acid

Glutamic acid is the most rapidly metabolized amino acid in the central nervous sys being incorporated into proteins and several other compounds (including small molec peptides) It also produces glutamine and, through decarboxylation, GABA, which is furt metabolized to succinate and via the tricarboxylic acid cycle to CO_2, the latter react being reflected in the relatively low recovery rate for glutamate in the brain homogena Thus in our experiments the label which was initially in the glutamate here might have partially located in several other compounds (glutamine, GABA, succinate semialdeh and succinate) by the end of the experiment GABA transaminase is located in the mi chondria (Waksman *et al* 1968 Bloch Tardy *et al* 1974) and interestingly the concent tion of [^{14}C]glutamate or compounds derived from it in the mitochondria was low in t present case, indicating its rapid metabolism here. Both glutamate glutamine and GAB as well as some peptides containing glutamate, occur in synaptic vesicles (Lähdesmäki et 1977 a, Lähdesmäki and Winter 1977), and glutamate has specific transport systems b in synaptosomes (Lähdesmäki *et al* 1975 1977 b) and synaptic vesicles (Lähdesmäki et

a) and also specific receptor sites at the synaptic membranes (Michaelis *et al.* 1974, ...enmäki *et al.* 1977 b). There is strong evidence that glutamate is an excitatory neuro-...mitter in the central nervous system (Curtis and Johnston 1974).

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From August Krogh Institute, University of Copenhagen, Denmark

Interrelationships between skeletal muscle adaptations and performance as studied by detraining and retraining

By

Michael E. Houston, Henning Bentzen and Henrik Larsen

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Abstract

uston, M. E., H. Bentzen and H. Larsen. *Interrelationships between skeletal muscle adaptations and performance as studied by detraining and retraining.* Acta physiol. scand. 1979 *105* 163–170.

effects of 15 days of detraining and 15 days of retraining were studied in 6 well-trained runners. Detraining resulted in significant decreases in the mean activities of succinate dehydrogenase (SDH) and lactate hydrogenase (LDH) of 24 % and 13 %, respectively but no significant increases in these enzyme activities ured with retraining. Maximal oxygen uptake ($\dot{V}O_2$ max) decreased by 4 % with detraining ($p < 0.05$), increased by a similar amount with retraining. Performance time in an intense submaximal run decreased by 25 % ($p < 0.05$) with inactivity but still averaged 9 below the initial level after retraining. Submaximal heart rate and peak heart rate during the performance run were higher after detraining by 4 and 9 ats per min, respectively ($p < 0.05$). With retraining, these heart rate values were decreased by 7 and 9 ts per min ($p < 0.05$). Blood lactate concentrations after the $\dot{V}O_2$ max and performance run were approximately 20 lower after detraining and retraining ($p < 0.05$). Muscle fibre areas for three subjects ded to be larger in biopsy samples taken after detraining and retraining. These data suggest that even rt periods of detraining result in significant changes in indices of physiological capacity and function subjects near their upper limit of adaptation, and that a longer period of retraining is necessary for scle to re-adapt to its original trained state.

words: Adaptation, detraining, retraining, muscle enzymes, oxygen uptake, performance.

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ers who have undergone a prolonged endurance training stimulus for a number of years ow marked adaptations in the oxygen transport system (Ekblom and Hermansen 1968, llock 1977) and in the capacity of the trained skeletal muscles for oxidative metabolism ltin *et al.* 1977). When compared to untrained control subjects, however elite distance nners have maximal oxygen uptake ($\dot{V}O_2$ max) values that are approximately two fold eater (Gollnick *et al.* 1972, Pollock 1977), but oxidative enzyme activities (e.g. SDH) that ay be up to 4 times greater (Gollnick *et al.* 1972, Fink *et al.* 1977). There are other characteristics of trained skeletal muscle besides elevated oxidative enzyme activities. The pillary supply is enhanced in endurance trained muscle (Brodal *et al.* 1977, Nygaard and Nielsen 1978), an adaptation that has been shown to be related to training stimulus (Andersen and Henriksson 1977). Further in two recent studies, the cross sectional area of muscle

fibres from swimmers (Nygaard and Nielsen 1978) and runners (Jansson and Kaijser 19 have been reported to be smaller than those found in untrained people, although d from several earlier studies do not show this (Edström and Ekblom 1972, Gollnick et 1972).

Short term interruptions of year-long continuous training are often experienced by w trained athletes, but the circulatory and metabolic consequences of such short period inactivity are not known. This subject has been addressed by Booth (1977), at least fro theoretical perspective, and in two recent studies (Örlander *et al* 1977 Henriksson Reitman 1977) However these latter two investigations employed subjects, who ha relatively brief (7–10 weeks) training, were sedentary and therefore could hardly be cons ered as having approached their limit of adaptation.

In the present study male runners at the peak of their training were tested before after a 15 day period with no training stimulus, and then were re-examined after a subseq 15 days of retraining. Oxygen transport capacities, skeletal muscle characteristics as wel performance capabilities were tested in order to gain an insight into the plasticity of limiting adaptations to the absence and re-application of a training stimulus.

Material and methods

Subjects. 6 male runners volunteered to participate in this investigation after bei g fully informed of nature and risks of the procedures involved. They averaged 32.5 ± 2.8 (± S.E.) years of age, 63.2 ± 1 in weight and 176 ± 3 cm in height. Four of the subjects were distance runners who had trained year for at least 4 years, whereas the remaining two subjects ran shorter distances (400–1 500 m), but for t months preceding these experiments trained in a simila manner to the distance runners. Training sisted of long (15–30 km) continuous runs each day at a submaximal intensity (ca 75 % or more of $\dot{V}O_2$ The subjects employed in this study were considered to have approached, if not reached their lim adaptation and all the subjects had run the marathon (42.2 km) in times ranging from 2.58–3.17 h

General protocol The subjects were initially tested at the peak of their training (before detraini During the next 15 days, no training was permitted, and for the first 7 days of the detraining per walking plastc cast was placed on the right leg, immobilizing the muscles of the calf For the remain days of detraining, the subjects perf rmed their normal daily activities except that no strenuous ph activity was permitted Following the second testing period (after detraining, A) the subjects comm training, attempting to ncrease the duration nd ntensity of training as rapidly as they felt possible 15 days f retraining the subjects were re-tested (after retraini g, R). Only in the latter half of the retr period were the subjects ble to tolerate their previous traini g distances.

As the subjects were fearful of the negative ffects of detraining on thei future performance only (15 day) period was possible The casting was used to enhance the effects of a withdrawal from the training stimulus. No dverse effects from this procedure were experienced by any of the subjects subsequent running tests.

Testing procedures Testing took place on two consecutive days for each of the phases of the On the first day the subjects reported t the laboratory and a percutaneous needle biopsy (1962) was made in the lateral head f the right gastrocnemius muscle Following the biopsy a ma oxygen uptake ($\dot{V}O_2$ max) test was performed on a motor driven treadmill. The treadmill speed 16.1 km h^{-1} and after 4 min at 0 %, the grade was increased by 2 % increments every 2 min till exha (ca. 10 min). On the second d y the subjects performed treadmill run to exhaustion at 16.1 km h at a grade calculated to demand 90 of the initial $\dot{V}O_2$ max. For subseque t testing the same spe grade for each subject was employed. The time to exhaustion in this submaximal run was taken to be endurance performance capacity and during these runs the subjects were unaware of thei actual time. During the two treadmill tests expired air was collected in Douglas bags, and its volume in a Tissot spirometer and analyzed for O_2 nd CO_2 fractions using a Ser LB-2, respectively Heart rat was determined from ECG tracings made at

ng tests. At the end of the treadmill tests blood sample was obtained from superficial arm vein analysed for lactate content using fluorimetric procedure (Lowry and Passonneau 1972).
muscle sample analyses. One portion of muscle tissue from each biopsy sample was quickly frozen in liquid nitrogen and stored at −80°C for later enzyme analyses. The remaining muscle sample was mounted embedding medium, frozen in isopentane cooled in liquid nitrogen, and stored at −80°C until serial cross sections (10 μm) were cut at −20°C in microtome. The muscle fibres were classified on of the myofibrillar ATPase reaction at pH 9.4 (Padykula and Herman 1955) using the preincubation techniques described by Brooke and Kaiser (1970). On this basis, fibres were identified as types ST and FTb (Saltin *et al.* 1977). For the determination of muscle fibre composition 297 (180–478, mean range) fibres were counted in each biopsy sample.
scle capillaries were identified using the histochemical amylase periodic acid Schiff (amylase-PAS) nique of Andersen (1975). Where a capillary was cut longitudinally it was counted as one at each cell lary. The capillary density (capillaries per mm²) was obtained by outlining an area of muscle cross n, without artifacts, on a photograph (magnification 150X) of an amylase-PAS stained section. The ed area was determined by planimetry and the number of fibres within that area (approximately 100) ll as the number of capillaries belonging to the area were counted, and the ratio determined. The er of capillaries around fibres was obtained by determining the mean number of capillaries on the lary of at least 25 fibres of specific type. The fibre type area per capillary was calculated by dividing mean area of each fibre type by the mean number of capillaries bordering that fibre type. These procedures for determining muscle capillarization have been discussed in detail by Andersen and Henriksson). The mean area of at least 25 fibres of each type was determined with planimeter from photographs of sections stained with the amylase-PAS reagents. Using this technique, the variation in absolute for ST and FT fibres within duplicate samples from the same muscle (vastus lateralis) for 29 subjects ged 15 7 (Halkjær Kristensen and Ingemann-Hansen, personal communication).
muscle samples for enzyme analyses were homogenized at 1:100 dilution in an 0.1 M triethanolamine er, pH 7.6 and containing 0.05 bovine serum albumin and 5 mM 2-mercaptoethanol. Total phosphorylase and succinate dehydrogenase (SDH) activities were determined fluorometrically using procedures ribed by Conlee *et al.* (1976) and Essén *et al.* (1975), respectively. Lactate dehydrogenase (LDH) was yed fluorometrically in the direction of pyruvate reduction. An aliquot of the homogenate was added ml of reagent solution pH 7.0 containing imidazole, 20 mM sodium pyruvate, 1 mM bovine serum min 0.02%, and reduced nicotinamide adenine dinucleotide (NADH), 0.01 mM. All enzyme analyses each subject were determined on the same day and enzyme activities were expressed on the basis of wet scle weight.
Statistics. The significance of differences between conditions was tested using the paired t-test. Differences between conditions were considered significant at the 95% confidence level.

Results

$\dot{V}O_2$ max test. Individual data and mean values obtained during the $\dot{V}O_2$ max test are strated in Fig. 1. Detraining resulted in a decrease ($p < 0.05$) in $\dot{V}O_2$ max of 0.17 l min^{-1} (4–7.3), and retraining produced a significant increase of 0.15 l min^{-1} (0.5–6.6%). As notable body weight changes occurred in the group of subjects during the two phases the experiment, similar changes with detraining and retraining were obtained when $\dot{V}O_2$ max was expressed on a relative basis (ml kg^{-1} min^{-1}). Maximal expiratory volumes max) followed the same pattern as $\dot{V}O_2$ max during the experiment as is evidenced by absence of significant differences among the V max, $\dot{V}O_2$ max ratios for the three test riods (Fig. 1). Mean maximal heart rates (HR max) recorded during the $\dot{V}O_2$ max test creased by 4 beats per min (b min^{-1}) (1.5%, $p < 0.05$) when the training stimulus was moved. 15 days after commencing retraining, HR max decreased significantly by 7 b min^{-1} 7). After detraining mean lactate concentration in blood sampled immediately after treadmill maximum oxygen uptake test was 23% lower ($p < 0.05$) compared to the ... tion. No further changes in lactate concentration were noted following retraining.

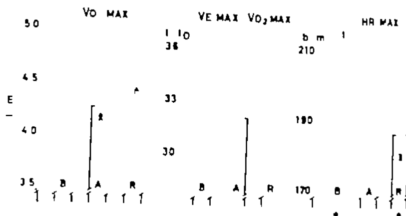


Fig. 1 Means and individual values for maximal oxygen uptake (VO_2 max), ventilatory equivalent ($\dot{V}_E$ VO_2 max) and maximum heart rate (HR max), before (B), after 15 days of detraining (A), and after days of retraining (R) for 6 well trained runners (the symbols ● and □ represent subjects who were trained for 400–1 500 m races). – significantly greater than after detraining (A) $p < 0.05$, ✣ significantly less than after detraining (A) $p < 0.05$

Performance run The work load employed in the treadmill run to exhaustion represented a mean energy output that was calculated to be 93.5 (91 9–95 2) % of the initial maximal oxygen uptake. No differences in mean oxygen cost for this run were noted for the times that the subjects were tested. Performance time (Fig. 2) decreased by 25 (50.5) % ($p < 0.05$) with detraining after retraining it was still 9 (−32 to 30)% below initial time. The peak heart rate recorded during this run increased by 9 b min (1 10%) the test following detraining ($p < 0.05$), and in the subsequent test peak heart rate 9 b min⁻ lower (3–7 %, $p < 0.05$). Blood lactate levels obtained after the run were sig

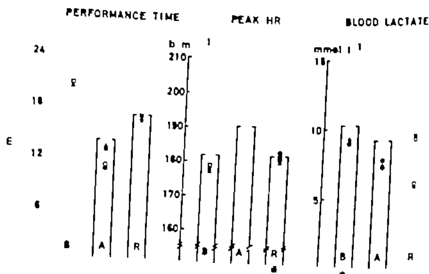
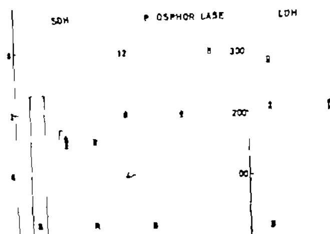


Fig. 2. Means and individual values for performance time in an intense submaximal run, peak heart rate (peak HR) measured during the run and blood lactate concentration measured after the run, before, after 15 days of detraining (A) and after 15 days of retraining (R) for 6 well-trained runners (the symbols ● and □ represent subjects who previously trained for 400–1 500 m races). – significantly different from after detraining (A) $p < 0.05$, ✣ – significantly greater than after detraining (A) and after retraining $p < 0.05$



Means and individual values for the activities of the enzymes succinate dehydrogenase (SDH), horylase and lactate dehydrogenase (LDH) in the lateral part of the right gastrocnemius muscle (B) after 15 days of detraining (A) and after 15 days of retraining (R) for 6 well-trained runners ymbols ● and □ represent subjects ho previously trained for 400–1 500 m races). = significantly than after detraining (A) and after retraining (R) $p < 0.05$.

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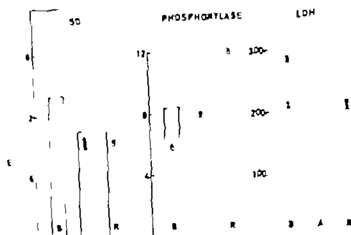
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ject	Fibre composition						Muscle fibre area μm² 10³					
	Type ST			Type FT			Type ST			Type FT		
	B	A	R	B	A	R	B	A	R	B	A	R
	64.7	63.9	69.1	35.1	36.1	29.5	3.75	4.00	4.84	3.84	4.06	4.56
	73.6	71.9	72.4	26.0	27.5	27.6	4.06	4.00	5.04	4.85	6.42	7.31
	52.2	53.2	51.8	47.8	46.2	44.0	4.35	5.56	4.70	3.92	5.18	4.78
	64.2	63.0	65.1	35.7	36.6	33.7	4.05	4.52	4.69	4.20	5.22	5.56

F g. 1 Means and individual values for maximal oxygen uptake ($\dot{V}O_2$ max), \eatlng VO max) and maximum heart rate (HR max), before (B), after 15 days of de days of retraining (R) for 6 well-trained runners (the symbols ● and □ represe trained for 400-1 500 m races). = significantly greater than after detraining (A less tha afte detraining (A) $p < 0.05$

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Fibre composition %						Muscle fibre area μm² 10³					
Type ST			Type FT			Type ST			Type FT		
B	A	R	B	A	R	B	A	R	B	A	R
66.7	63.9	69.1	33.3	36.1	29.5	3.75	4.00	4.84	3.84	4.06	4.56
73.6	71.9	72.4	26.4	27.5	27.6	4.06	4.80	5.04	4.85	6.42	7.31
52.2	53.2	53.8	47.8	46.2	44.0	4.35	3.56	4.70	3.92	5.18	4.78
64.2	63.0	65.1	35.7	36.6	31.7	4.05	4.32	4.60	4.20	5.22	5.56

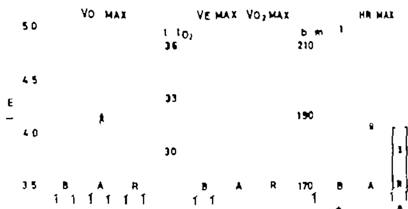


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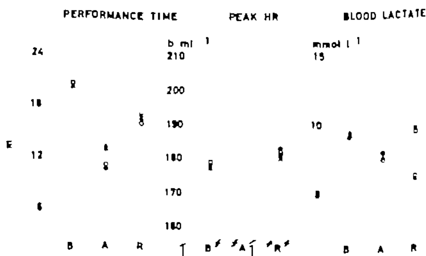
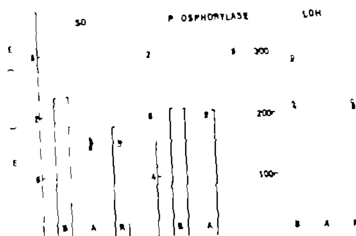


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	B	A	R	B	A	R	B	A	R	B	A	R
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	73.6	71.9	72.4	26.0	27.5	27.6	4.06	4.00	5.04	4.85	6.42	7.31
	52.2	53.2	53.8	47.8	44.2	44.8	4.35	5.56	4.70	3.92	5.18	4.78
	64.2	63.0	65.1	35.7	36.6	33.7	4.05	4.52	4.69	4.20	5.22	5.56

TABLE II Muscle capillarization before (B) and after (A) detraining and following retraining (R) ... subjects.

Subject	Capillaries per mm²			Capillaries around fibres						Fibre type area per capillary (...					
				Type ST			Type FTa			Type ST			Type FTa		
	B	A	R	B	A	R	B	A	R	B	A	R	B	A	R
MH	580	530	570	5.5	5.8	7.0	5.5	5.6	6.8	0.69	0.69	0 62	0.70	0 73	[illegible]
KR	535	555	481	6.1	6.7	6.7	5 8	8 1	6.9	0.67	0.60	0.75	0 84	[illegible]	[illegible]
SK	559	484	500	6.5	6.6	6.5	6.4	6.4	6.5	0 67	0.84	0.72	0 61	[illegible]	[illegible]
Mean	558	523	517	6.0	6.4	6.7	5 9	6.7	6.7	0.67	0.71	0.70	0 72	[illegible]	[illegible]

5.5 ± 3 6 % for type ST FTa and FTb fibres, respectively Only the two subjects who previously specialized in training for shorter distances had any FTb fibres (13.3 and 19... and thus further data for these fibres is omitted. The mean cross sectional area for ST f... was 4.58 ± 0 41 10³ μm and for the FTa fibres, was 5 14 ± 0 59 10³ μm There ... 525 ± 71 capillaires mm⁻² and in terms of diffusion, 690 ± 86 μm of ST fibre area ... provided by one capillary the corresponding value for FTa fibres was 780 ± 134 μm ... capillary

For the 3 subjects who had been training the longest (approximately 8 years) we ... able to obtain complete data on muscle morphology over the course of the experiment ... is illustrated in Table I and II No significant changes in fibre composition were obs... during the two phases of the study although there were some few FTb fibres, represent... approximately 0 6% of the total fibre population in the second (A) and third (R) h... chemical sections for two of these subjects. The mean areas for ST and FTa fibres in m... samples obtained after detraining and retraining tended to be larger compared to ... values. However only for the FTa fibres were the changes in fibre areas greater than ... expected experimental error Muscle capillarization (Table II) also appeared to be al... with inactivity and subsequent training. In 2 of the 3 subjects, capillary densities (capil... per mm²) were smaller in the second (A) and third (R) biopsy samples.

Discussion

Although the detraining and retraining periods were brief significant changes occurr... maximal oxygen transport capacity performance time and muscle enzymes.

Muscle enzymes Before detraining, the present subjects had muscle SDH activities ... were approximately two times higher than is found in untrained males of almost the s... age (13.9 *vs* 7–8 μmol g⁻ min) but phosphorylase and LDH activities that were ... the range found for the untrained, 8 4 *vs* 8–12 and 256 *vs* 200–300 μmol g min ... spectively (unpublished data from this laboratory) The change in the activities of the ... acid cycle enzyme SDH and the glycolytic enzyme LDH but not the glycogenolytic en... phosphorylase would indicate that the activities of the former enzymes in well-tra... muscle are dependent in part on the presence of an endurance training stimulus.

Muscle fibre area and capillarization The tendency to increased fibre areas with detra...

subjects was surprising. The initial fibre areas for these subjects were comparable to values reported by Jansson and Kaijser (1977) for orienteers, and smaller than the mean reported by these authors for 62 control subjects, that is 4.84 and 5.27 10^3 μm for ST and FTa fibres, respectively. It should be pointed out that although the present data and reported by Jansson and Kaijser (1977) and Nygaard and Nielsen (1978) reveal that athletes who have undergone prolonged endurance training have small muscle fibres, other studies (Edstrom and Ekblom 1972, Gollnick *et al.* 1972) do not show this. However, a physiological consequence of small fibre areas is that diffusion distances for oxygen and other nutrients are reduced. The capillary supply in skeletal muscle has recently been shown to increase in response to exercise training (Andersen and Henriksson 1977) and electrical stimulation (Brown *et al.* 1976). Although only 3 subjects were studied, the present data could suggest that muscle capillarization may be decreased with short detraining. However, further studies are clearly indicated.

Maximal oxygen uptake. The dissociation of changes in whole body oxygen transport capacity from the changes in metabolic oxidative capacity is clearly apparent in this study. The decline in SDH activity with detraining was more than 5 times that for VO max. Furthermore, retraining increased maximal oxygen uptake to nearly pre-training values while SDH activity was only marginally increased. These results are in close agreement with those of Henriksson and Reitman (1977) and Örlander *et al.* (1977), and reinforces the latter's contention that changes in VO_2 max need not be accompanied by corresponding changes in muscle enzymes.

Performance. The decrease in performance time was of approximately the same relative magnitude as the decrease in SDH activity after detraining. With this in mind, it is tempting to suggest a causal relationship between muscle oxidative capacity as reflected by SDH activity and performance time in the intense submaximal run. However, the corresponding changes for these two variables after retraining are not related. Rather, the decrease in performance time with detraining and the subsequent increase to nearly initial values with retraining are more in accordance with changes in maximal oxygen uptake. At least these two show relative changes that more closely match each other in the direction of change. It may be that the work load selected for the performance measure was too intense to demonstrate a closer relationship with muscle oxidative capacity.

Practical implications. It is not uncommon for man to undergo forced inactivity for brief periods of time as a result of minor injuries. The results of the present study are useful for illustrating the type of changes that could be expected to occur for endurance trained persons. Although the experimental design precludes differentiating between the effects due to the absence of training and those of the casting, some generalizations can be made. First, the inability of the subjects to completely attain initial performance levels following retraining was not very striking. On an average, performance times following retraining were only by 9 % lower than the initial times and the variation was considerable. The results of this study demonstrate that short lasting detraining (inactivity) leads to moderate reductions in VO_2 max, and to relatively greater reductions in submaximal performance times and activities of SDH and LDH. During retraining performance time and in particular VO max, are re-established faster than SDH activity.

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...anscapillary passage of albumin, effects of tissue cooling and of ...increases in filtration and plasma colloid osmotic pressure

By

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Abstract

B A. KAMIYA and B. FOLKOW *Transcapillary passage of albumin, ...ffects of tissue ...ooling and of increases in filtration and plasma colloid osmotic pressure* Acta physiol. ...cand 1979 *105* 171 187

...e clearance of radiolabelled serum albumin was measured in the perfused, maximally vasodilated ...vascular bed of rat hindquarters during tissue cooling, during increases in filtration and during ...s in serum colloid osmotic pressure. Albumin clearance during ordinary serum perfusion at iso-...etry amounted to 0.03 ml/min 100 g, increasing linearly ...th filtration rate to some 0.07 ml/100 g at 0.3 ml/min 100 g of filtration During cooling from 36°C to 14°C both CFC and 'initial ...n clearance at isogravimetry decreased some 40%, in due proportion to the increased viscosity of ...nd increases of the colloid osmotic pressure of the perfusate correspondingly increased both the ...mean capillary pressure and 'initial' albumin clearance during isogravimetry — It is concluded ...at during isogravimetry the transmicrovascular albumin passage is to about 70 per cent due to ...on, and only some 30 per cent of transport at ordinary serum colloid osmotic pressures takes place ...ffusion, both events presumably via "large pores" There was no evidence that transendothelial vesicular ...ort should to any significant extent contribute to the passage of albumin from vessels to tissue.

...e mechanisms behind the transfer of macromolecules across microvascular walls are ...incompletely understood and a matter of much controversy While diffusion through ...l pores is acknowledged as the main principle for transcapillary passage of small ...ophilic substances (*cf* Landis and Pappenheimer 1963, Crone and Lassen 1970), this ...s the case for macromolecules Grotte (1956) postulated the existence of large capillary ...s ('leaks') through which macromolecules could pass by bulk filtration and/or diffusion. ...ation was proposed to be the dominating macromolecular transport mechanism by ...rrow group (*cf* Shirley *et al.* 1957) and later by Haddy Scott and Grega (1972, 1976), ...m, Parving and Rossing (1974) and Rutili (1977).

...owever particularly in recent years the interest for endothelial micropinocytosis as a ...able major route for transcapillary macromolecular passage has been much increased, ...ups mainly because of the often suggestive electronmicroscopic illustrations of rapid ...er passage into the interstitial space and the simultaneous presence of tracer-labelled

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g isogravimetric conditions capillary filtration and absorption are in complete balance, though filtration dominates at the arteriolar end and absorption at the venular end, causing 'paracapillary circulation' (Landis and Pappenheimer 1963).

If the capillary membrane were homoporous there would be no net solvent drag effects during isogravimetry, net transport being then exclusively dissipative in nature. However, according to the two-pore theory (cf. Grotte 1956) macromolecules pass almost exclusively through a small number of large pores (r ≈ 250 Å), probably located at the venular end of the capillaries (e.g. Wiederhielm 1968). In such pores the reflection coefficient for albumin is likely to be near zero, so there should here be only a negligible 'effective' colloid osmotic pressure gradient to balance off the local hydrostatic pressure gradient. Albumin and other proteins will continuously pass out through the large pores as long as the local hydrostatic pressure exceeds that in the tissues, and also pass out by diffusion in proportion to the overall difference in protein concentration between perfusate and tissue fluid. At isogravimetry this bulk flow of fluid through the large pores is, however, balanced off by osmotic absorption of solvent through small pores and across endothelial cells, from which macromolecules are excluded (cf. Grotte 1956). Such a type of isogravimetric 'volume circulation' in the capillary membrane thus includes a substantial unidirectional solvent drag transfer of macromolecules from blood to tissue, for which theoretical descriptions have been presented (e.g. Kedem and Katchalsky 1963, Patlak and Rapoport 1971, Perl 1973).

In the present study the simplest of these derivations will be tested, i.e. that by Kedem and Katchalsky as modified by House (1974). To simplify the situation it was assumed that the reflection coefficient for the proteins was zero in the large pores, while it was 1 for small pores and endothelial cell membranes. The following expression is then obtained for solute flux, F_p, of albumin (mg/min · 100 g of tissue) and its clearance (CL, ml/min · 100 g of tissue) (for derivation see appendix I):

$$F_p = C_p CL = PS(C_p - C_i) + \sigma(1 - \sigma) CFC \bar{C} \Delta\pi + (1 - \sigma)\bar{C} F \qquad \text{Eq (2)}$$

C_p is the tracer concentration in plasma (mg/ml), C_i the interstitial tracer concentration (mg/ml), PS the diffusional permeability of albumin through large pores (ml/min · 100 g of tissue), σ is the overall, weighted osmotic reflection coefficient for serum proteins to the capillary membrane as a whole, which is assumed to be approximately equal to the weighted solvent drag reflection coefficient (see Durbin ...), CFC the capillary filtration coefficient (ml/min · mmHg · 100 g tissue), $\Delta\pi$ the colloid osmotic pressure difference across the capillary membrane (mmHg), $\bar{C}$ the mean tracer concentration in the large pores (mg/ml). Finally, F represents the net filtration rate (ml/min · 100 g tissue).

The first term to the right in Eq (2) represents albumin diffusion through the large pores, the second the isogravimetric solvent drag transport through the large pores and the last one albumin convectional transport coupled to net filtration or absorption. The last-mentioned two terms are derived from the pressure dependent term of Eq. 1-9 (see appendix I). The isogravimetric solvent drag term was here derived under the simplified condition of free convection of albumin and most other serum proteins through large pores and their complete exclusion from small pores and cell membrane pathways. In reality the isogravimetric solvent drag might be lower because the reflection coefficient for serum proteins in large pores may exceed zero. Further, the large pores are probably located at the venular ends of the capillaries where hydrostatic pressure is lower than at the capillary midpoint. The inhomogeneous distribution of large pores is thus intrinsically inherent in σ, which represents a weighted mean of all partial reflection coefficients for serum proteins in relation to the different porous structures in the capillary membrane.

With all its limitations, this formula is interesting since it shows how albumin clearance at isogravimetry depends partially on the transcapillary colloid osmotic pressure difference ($\Delta\pi$) and also on CFC. Thus, if isogravimetric capillary pressure is increased by raising π_p, the isogravimetric bulk flow of proteins through large pores increases, as does also the osmotic solvent absorption through small pores and endothelial cells. The isogravimetric 'volume circulation' is thus increased and so is the flux of protein from blood to tissue. As protein clearance by diffusion is independent of concentration, its increase in this situation is not coupled to the elevated plasma protein concentration *per se* but only to the increased isogravimetric 'volume circulation'. Further, if CFC is decreased, e.g. by increased fluid viscosity as during cooling, albumin clearance should decrease in proportion to the decrease in CFC and in isogravimetric 'volume circulation' in the capillary membrane.

Arising from these considerations it is evident that independent changes of tissue temperature, of capillary filtration-absorption and of plasma colloid osmotic pressure could be used to discriminate semi-quantitatively between dissipative and convective processes involved in albumin transport across microvascular membranes. Vesicular transport may here be included among dissipative mechanisms (cf. Renkin 1977), and by means of tissue cooling during isogravimetry or net filtration (cf. Rippe, Kamiya and

micropinocytosic vesicles (e.g. Bruns and Palade 1968). Thus, towards the background of such morphologic observations and calculations based on Grotte's experimental data, Winne (1965) concluded that macromolecular transfer occurs either by transendothelial vesicular transport or by large pore filtration with only little contribution by diffusion. In contrast Renkin and coworkers (cf. Renkin 1964, Garlick and Renkin 1970, Carter and Joyner 1974) considered vesicular transport and large pore diffusion as predominant, but have recently included also large pore filtration, at least during congestion (Renkin et al. 1977). Thus, opinions concerning capillary macromolecular transport differ considerably between laboratories, presumably reflecting different methodology and the many technical difficulties involved, as in lymph studies (cf. ... et al. 1977).

Therefore, the aim of the present study was to evaluate to what an extent diffusion, convection and vesicular transport contribute to the transvascular albumin passage, in the maximally vasodilated isogravimetric rat hindquarter preparation as a model (Folkow et al. 1974), since it has proved suitable also for quantitative vascular and capillary studies (Rippe and Stage 1978, Rippe, Kamiya and Folkow 1978). After ligation of the lymph drainage the preparation was perfused under well controlled hemodynamic conditions at constant flow with horse serum, using radiolabelled albumin as macromolecular tracer. The plasma clearance of tracer albumin could be estimated 1) during tissue cooling, which should greatly depress active transport mechanisms, 2) during changes in venous pressure and hence in filtration, 3) during isogravimetry but at increased plasma colloid osmotic pressures, implying that mean isogravimetric capillary pressure is correspondingly increased. The effect of tissue cooling on the transvascular albumin passage was earlier investigated in paired experiments at equal levels of induced filtration, using a perfusate of subnormal colloid pressure (Rippe, Kamiya and Folkow 1977). These initial results suggested that capillary micropinocytosis is quantitatively unimportant or even irrelevant as a transport mechanism for large molecules across capillary walls.

Theoretical Considerations

Solvent drag effect on large molecular transport by means of isogravimetric volume circulation in the capillary membrane

The theory of irreversible thermodynamics (Kedem and Katchalsky 1958, 1961, 1963) has recently been applied also for the analysis of transcapillary passage of large molecules (e.g. Perl 1975, Blake and ... 1976, Renkin et al. 1977). However, even though the data were interpreted in terms of two-pore or multi-pore capillary models, the calculations were generally based on homogenous one-pore model, as ... out by Brace, Granger and Taylor (1977). In the present investigation the two-pore capillary model, as postulated by Grotte (1956), was tested applying the simplest derivations concerning irreversible thermodynamics (Kedem and Katchalsky 1963, ... 1974).

According to the Starling hypothesis the capillary fluid equilibrium is a function of the balance between the capillary to tissue hydrostatic pressure gradient $(P - P_t)$ and the opposing colloid osmotic pressure gradient $(\pi_p - \pi_t)$. However, for membranes not perfectly semipermeable this theory should be modified by taking into account the Staverman reflection coefficient (Staverman 1951). According to the theory of irreversible thermodynamics the transcapillary fluid flux is then characterized by:

$$F = CFC[(P - P_t) - \sigma(\pi_p - \pi_t)]$$

where F is the volume flux (ml/min · 100 g of tissue), CFC is the hydraulic conductivity (ml/min · mm Hg · 100 g) and σ the weighted Staverman osmotic reflection coefficient for the colloid, which is ...

...al albumin clearance at markedly elevated venous pressures ($P_v > 40$ mmHg)

...pairs, perfused at equal temperatures (36–37°C), venous pressure (P) in the test preparation was ...ually elevated to 40–70 mmHg while P in the control was kept between 3 and 34 mmHg. During the perfusion period the oxygenated heparinized horse serum was diluted to 60 with Tyrode solution ...ther enhance the filtration, ...hile undiluted horse serum was used during the tracer free perfusion to the preparations isogravimetric under these conditions.

...ual albumin clearance at different perfusate protein concentrations at normal ...erature (36–37°C) and during tissue cooling to 13–15°C

...lding bovine albumin (Fraction V powder Sigma Chemical Company USA) to normal horse serum, ... protein concentration was increased from 61 g/l to 111 g/l in two paired experiments and to 86 g/l ... In three additional perfusion experiments horse serum, diluted to protein concentration of 36 g/l, ...ised. In each of these experiments one of the paired preparations was cooled as described above.

Data processing and calculations

... rationed ...bove the interstitial accumulation of radiolabelled albumin and of tissue fluid was measured ... standardized period, usually one hour of tracer perfusion, in order to reach high interstitial tracer ...ntrations for the measurements, and during net filtration, ...hile escape of fluid via lymphatics ...as ...ed (cf. Rippe and Folkow 1977). As earlier observed in similar preparations at moderate constant ...ltration (0.05–0.3 ml/min 100 g) (e.g. Appelgren, Jacobsson and Kjellmer 1966, Friedman 1976), ...terstitial accumulation of tracer albumin occurs almost linearly during periods up to 1 h. The intersti... protein concentration is determined by the current balance between "large pore filtration (as ... ...tricted passage of plasma) and "small pore filtration (largely an ultrafiltrate of plasma), which depends ...e filtration rate and, in turn, on the capillary pressure. Therefore at certain rate of constant filtra... in the present study around 0.08 ml/min 100 g during horse serum perfusion) these two processes, ... owing concentration and the other dilution of interstitial proteins, tend to cancel out so that the ... albumin accumulation becomes ...ith time perfectly linear function.

...this particular rate of filtration the albumin clearance will after 1 h of tracer perfusion be the same as ... the tracer perfusion started, and thus reflect the "actual" albumin clearance. During isogravimetric ...ditions, however, there will be continuous rise of interstitial colloid concentration with time and the ...l albumin clearance value ...ill therefore be underestimated by the one hour net clearance value ...hile ...cause of continuous dilution of interstitial colloids, is overestimated after marked filtration. This is ...ent from the theoretical analysis of the transient process of interstitial tracer accumulation, shown in ...ndix II. Based on these derivations the 1 h net clearance values were corrected to "actual" clearance ... in the following ...ay. First the 1 h net albumin clearance value CL (ml/min 100 g initial tissue ...) ... in every experiment calculated from the mean value of the six determinations of muscle tracer ...ent I_t (CPM/100 g of initial tissue mass), per unit time, (min) and plasma tracer concentration, ...PM ml of plasma):

$$CL = \frac{I_t}{tC_p} \qquad \text{Eq (3)}$$

...g isogravimetry the relation between the initial clearance, CL_1 and CL was calculated from:

$$\frac{CL}{CL_1} = 1 - (\sigma p/\Delta\pi)(CL\ t/2V_o) \qquad \text{Eq (4)}$$

...ere σ_p as earlier defined, $\Delta\pi_o$ denotes Δ (see above) at time zero, and where V denotes the initial ...titial fluid volume.

... calculated according to Landis and Pappenheimer (1963), π_p as for undiluted horse serum estimated ...5 mmHg, for 60 diluted horse serum to 10.5 mmHg, for the perfusate containing 86 g protein/l ...mmHg and for that containing 111 g protein/l to 60 mmHg. π was according to recent measurements ... muscle (Aukland, personal communication) set to 8 mmHg, corresponding to an interstitial protein ...entration around 40–50% of that in plasma (cf. Creese, D'Silva and Shaw 1962, Rutili and Arfors ...Fadnes and Aukland 1977). This ...grees well ...ith indirect π_i estimations in cat muscle (Eliassen ... 1974), of P is around zero. V for albumin distribution ...as set at 10 ml/100 g muscle as deduced ...hole body measurements of the interstitial media space (e.g. Ladegaard-Pedersen and Engell 1972),

Folkow 1977) one would be able to discriminate between this active endothelial mechanism and [illegible] since the latter should be f r less temperature-sensitive than microvesicular processes (see below). Consequently the present investigation was designed according to these principles, as also [illegible] Methods.

Methods

General arrangement

Experiments were performed on 22 matched pairs of isolated hindquarters from male Wistar [illegible] weighing between 260 and 350 g. The preparation techniques as well as the general technical [illegible] and radioactive assay have earlier been described in detail (Rippe and Folkow 1977). Briefly [illegible] ments were designed as paired, parallel perfusions of one test hindquarter preparation and one [illegible] using oxygenated, heparinized horse serum (Normal serum, SBL, Sweden) as perfusate, to [illegible] added radiolabelled albumin (^{125}I-albumin Radiochemical Centre, Amersham) as macromolecular [illegible] In the two animals all factors but the test parameters were kept as constant and equal as possible. [illegible] vasodilatation was induced and maintained throughout by papaverine infusion, 10–20 µg [illegible] Continuous recordings were performed concerning mean arterial pressure (P_A in the tail artery) [illegible] pressure (P_V, in caval side branch), flow rate (8–12 ml/min), total weight and tissue temperature [illegible] preparations, which were arranged for the isogravimetric technique (Pappenheimer and Soto Rivera [illegible] Rippe and Stage 1978).

Subsequent to the period of tracer perfusion, which usually lasted 60 min, the vascular tree was freed from tracer d rin g 8 min by shifting to tracer free perfusate Three pairs of standardized muscle samples (*cf* Rippe and Folkow 1977) were then dissected out from each preparation, weighed and [illegible] with respect to tracer content in a well scintillation counter after which they were dried and [illegible] Perfusate content f free ^{125}I was estimated by precipitating the perfusate with 10 % TCA and [illegible] supernatant. It averaged 1 6 of total perfusate radioactivity Correction for free iodine [illegible] however not deemed necessary because free iodine is distributed extracellularly and should then be [illegible] washed out during the 8 ml of final tracer free perfusion.

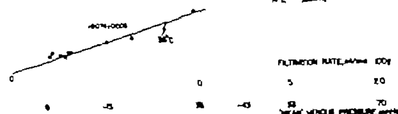
Tissue fluid accumulation in the musculature was followed by continuous weighing of the rat hindquarter on a balance connected to a force transducer (Grass FT 10C) writing on a Grass polygraph. Total increase during the perfusion period, as corrected f the skeleton (8 % of the hindquarter weight) related well with the calculated weight ncrease of the muscle samples. The latter values were [illegible] from the relation between wet and dry weights, as compared to corresponding samples from [illegible] controls (Rippe and Folkow 1977).

Determination of perfusate protein conce trations was performed according to the method [illegible] Vickers M300 and by paper lectrophoresis. The ratio between albumin and globulin concentrations in the perfusate needed f th colloid osmotic pressure estimations, as described under Data processing [illegible] calculations was determined by paper lectrophoresis.

Experimental procedure

1 *Initial albumin clearance during isogra imetry and moderate filtration t normal temperature (36–37° C) and during tissue cooling to 13–15° C*

Eight control preparations were perfused t 36°C in parallel with eight test preparations, reduced in temperature to 13–15 C by means of precooling the perfusate to just below 5 C. Two of these [illegible] were kept isogravimetric throughout, while the others were set to varying rates of filtration by pressure elevation up t 28 mmHg. I each pair the net filtration rate was kept constant throughout, checked by the weight recordings and sl ght adjustments f venous pressure were made whenever [illegible] Moreover filtration rates were i most periments set bout equal in both preparations. Temperatures were monitored by thermistors placed in fossa rectovesicalis in each hindquarter and [illegible] by two separat thermoequilibrators (*cf* Fig. 1 Rippe nd Stage 1978), setting the inflow temperature f the horse serum perfusat to the wished levels. Cooling of the test preparation from 36–37°C to [illegible] usually required some 15 min, during which period th preparations were perfused with tracer free [illegible] Before and after the cooling period, CFC (*cf* Eliassen *et al* 1974) was measured in four animals [illegible] maximal vasodilatation t check that CFC was reduced roughly in inverse proportion t the temperature dependent viscosity increase, as also bserved earlier (*cf* Rippe and Grega 1978). Otherwise CFC [illegible] ments were voided in order not to disturb the capillary fluid equilibrium in preparations kept isogravimetric or t low filtration rates (≤0 1 ml/min 100 g).



Initial albumin clearance during perfusion with horse serum, diluted to 60 with Tyrode, versus filtration rate and venous pressure. At venous pressures below some 40 mmHg there seems to be a linear relationship between albumin clearance at 36°C and filtration rate (= 0.97) with approximately the same regression coefficient (β) as for undiluted horse serum at 36°C. Note that the ordinate intercept is only ml/min 100 g for the diluted perfusate, because of the decreased 'isogravimetric' solvent drag transport of albumin at the lower serum colloid osmotic pressure. The regression line shown (continuous) calculated from the control values of the six paired experiments, in which the 'test' animals were subjected to markedly elevated venous pressures, and from the control values of the three paired experiments, in which the 'test' animals were perfused with cooled, diluted horse serum. First at venous pressures above mmHg there is a marked increase in albumin clearance, probably reflecting the "stretched pore phenomenon" (cf. Shirley et al. 1957).

significantly from each other (p 0.001), the ordinate intercepts being 0.0195 ± 0.003 ml/min 100 g and 0.0291 0.0020 ml/min 100 g, respectively. These values represent albumin clearance at *isogravimetry* in the cooled and control preparations during maximal dilatation. Their difference corresponds closely to the temperature-dependent difference in plasma viscosity which will to the same extent affect *isogravimetric large pore filtration*. At normal vascular tone the 'effective' exchange area for filtration is 3–4 times lower for diffusion around 4–5 times lower than during maximal vasodilatation (Rippe, and Folkow 1978). This would imply an albumin clearance value of 0.006–0.007 ml/min 100 g at ordinary resting vascular tone during isogravimetry and normal tissue temperature, in close agreement with *in vivo* values in rabbit skeletal muscle (Bill 1977) and hindpaw (Renkin *et al.* 1977).

Initial perfusate albumin clearance at markedly elevated venous pressure

60 horse serum in Tyrode was used as perfusate the initial albumin clearance extrapolated to isogravimetry during maximal vasodilatation was markedly lower 0.010 ± 0.004

which corresponds well with the albumin space, as deduced from the extravascular mass of interstitial albumin concentration (*cf* Lassen, Parving and Rossing 1974). This value on V is lower than the one recently obtained by Bell, Watson and Renkin (1978) in skeletal muscle, reflecting that the muscle interstitial fluid normally forms a variable and easily mobilized fluid depot cardiovascular system (Öberg 1964). Anyway a distribution volume for albumin substantially 10 ml/100 g of muscle is not compatible with the interstitial pressure-volume data of Eliassen et al. Using these values for τ_p, $\Delta\pi$ and V_e, CL_1^e can be calculated by Eq (4).

During constant net filtration the initial albumin clearance, CL can be calculated from the equation

$$CL = CL_1^e \left[\frac{\tau_E}{\Delta\pi}\ \frac{CL}{2F}\ \frac{\lambda}{1+\lambda} - \left(\frac{\tau_p}{\Delta\pi} - 1\right)\left(1 - \frac{\ln(1+\lambda)}{\lambda}\right)\right] + CL$$

Here λ equals the total filtered volume (F t), divided by the initial interstitial volume, V
By inserting the measured values for CL F t and the value for CL_1 (see above) the initial albumin clearance during net filtration, CL can be calculated.

Statistical evaluation was performed according to general parametric routines. Thus, linear regression analysis was carried out as described by Brownlee (1967).

Results

1 Initial perfusate albumin clearance during isogravimetry and filtration at normal temperature and during tissue cooling to 13–15 C

A significant correlation between initial albumin clearance and filtration rate was also found in both the cooled and control preparations, as shown in Fig. 1 The regression coefficients were $(6.33 \pm 1\ 14)\ 10^{-}$ ($\pm$ S E.) (dimensionless) and $(7.94 \pm 0\ 73)\ 10^{-4}$ respectively, no significant difference in slope between the two However the roughly parallel lines

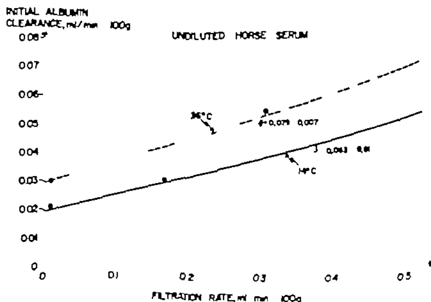
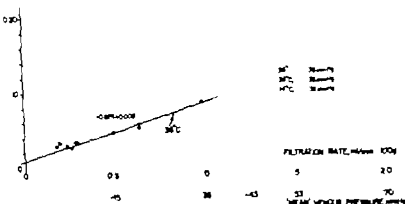


Fig. 1 Initial serum albumin clearance plotted against filtration rate at normal tissue temperature (broken line) and during tissue cooling to 13–15°C (continuous line). Open circles represent clearance values at 36°C and filled circles values at 14°C. Each point represents a mean value of 3 duplicate determinations in every experiment. The roughly parallell lines differ significantly from each other (p < 0 001), isogravimetric albumin clearance was decreased some 35% by cooling. The equations for the lines are y = 0.0291 + 0.0794 (r = 0.98) and y = 0.0195 + 0.0633 (r = 0 91).



[illegible] initial albumin clearance during perfusion [illegible] with horse serum, diluted to 60 [illegible] with Tyrode, versus filtration rate and venous pressure. At venous pressures below some 40 mmHg there seems to be [illegible] linear relationship between albumin clearance at 36°C and filtration rate ($r=0.97$) with approximately the same [illegible] coefficient (β) as for undiluted horse serum at 36°C. Note that the ordinate intercept is only [illegible] ml/min 100 g for the diluted perfusate, because of the decreased 'isogravimetric' solvent drag [illegible] of albumin at the lower serum colloid osmotic pressure. The regression line shows (continuous [illegible]) calculated from the control value of the six paired experiments, in which the 'test' animals were [illegible] to markedly elevated venous pressures, and from the control values of the three paired experiments, [illegible] the 'test' animals were perfused with cooled, diluted horse serum. First at venous pressures above [illegible] mmHg there is [illegible] marked increase in albumin clearance, probably reflecting the "stretched pore [illegible]" (cf. Shirley et al. 1957).

[illegible]significantly from each other (p [illegible] 0.001), the ordinate intercepts being 0.0195 ± 0.003 [illegible] 100 g and 0.0291 ± 0.0020 ml/min 100 g, respectively. These values represent [illegible] albumin clearance at isogravimetry in the cooled and control preparations during maximal [illegible]dilatation. Their difference corresponds closely to the temperature-dependent difference [illegible] viscosity which will to the same extent affect isogravimetric large pore filtration. [illegible] normal vascular tone the effective exchange area for filtration is 3–4 times lower [illegible] for diffusion around 4–5 times lower than during maximal vasodilatation (Rippe, [illegible] and Folkow 1978). This would imply an albumin clearance value of 0.006–0.007 [illegible] 100 g at ordinary resting vascular tone during isogravimetry and normal tissue [illegible]erature, in close agreement with *in vivo* values in rabbit skeletal muscle (Bill 1977) and [illegible] hindpaw (Renkin *et al.* 1977).

[illegible] perfusate albumin clearance at markedly elevated venous pressure

[illegible] 60 [illegible] horse serum in Tyrode was used as perfusate the initial albumin clearance extra[illegible] to isogravimetry during maximal vasodilatation was markedly lower 0.010 ± 0.004

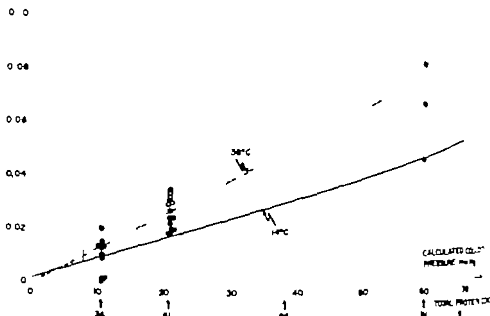


Fig. 3 Initial albumin clearance during isogra imetry plotted gainst calculated serum colloid o
pressure (cf Landis and Pappenheimer 1963) at protein concentrations of 111 g/l, 86 g/l and 61 g/l
with data obtained by e trapolation to isogra imetry of clearance alues t various filtration r
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8 mmHg (vertical hatched line) transcapillary colloid osmotic pressure difference is zero and the
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should be independent of the level of isogravimetric capillary pressure, was estimated to
ml/min 100 g.

ml/min 100 g, than when undiluted horse serum was employed (0 029 ± 0.002). The
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3 *Initial albumin clearance at different perfusate protein concentrations at normal temperature and during tissue cooling*

Using perfusates with different contents of serum protein, the initial albumin clear
values were plotted against perfusate colloid osmotic pressures, calculated accord
Landis and Pappenheimer (1963) Fig. 3 presents in such a diagram the albumin clear
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that also during isogravimetry albumin clearance increases with increasing perfusate c

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s also seen from Fig. 3 that during tissue temperatures just below 15°C albumin clear n reduced to about 60 per cent of that during normal tissue temperature of 36–37°C. se different temperatures the slopes of the linear regression for isogravimetric albumin nces as related to perfusate colloid osmotic pressure are (1.35 ± 0.09) 10^{-4} (r = 0.95) 0.80 0.10) 10^{-4} (r 0.90) respectively

Discussion

mechanisms behind the blood to tissue transport of macromolecules has been a matter ntroversy over the last few decades, where particularly the possiblity that micropino- s may be involved has aroused much interest. The evidence in support of such an e' cellular transport is mainly based on electron microscopic illustrations of tracers dily enter both pinocytotic vesicles and the interstitial fluid. However this fact by no means proves that the tracer is transported to the interstitial space *via* micro- ytosis, it may as well have arrived there by pore-bound filtration and diffusion.

: present investigation is an attempt to estimate in quantitative terms the relative ibutions of filtration, diffusion and esicular transport to the passage of plasma proteins the vascular bed to the interstitium, using labelled albumin as a tracer in the maximally lated, perfused rat hindquarter muscle vascular bed. The accumulation of this tracer of fluid in the muscle interstitial fluid space was measured during about one hour of r perfusion (*cf.* Rippe and Folkow 1977) in three different situations, *first* during e cooling, *second*, at varying filtration rates and, *third*, at different colloid osmotic ures of the perfusate. The obtained tracer flux data were mathematically treated to give s for actual' albumin clearance, according to derivations presented in appendix II.

ill be further discussed below the results of this study lend no support to the view microvesicular transport should be of any quantitative significance for the transcapillary age of macromolecules. Rather they strongly suggest that filtration and, to a minor nt, diffusion through large pores is in essence responsible for this macromolecular sfer also during isogravimetry This conclusion is based on the following findings 1) ng tissue cooling albumin clearance was reduced in close proportion to the temperature- ndent increase of perfusate viscosity and the related decrease in capillary filtration cient, also during isogravimetry 2) Tracer albumin clearance increased largely in ortion to increases in filtration rate, 3) Also during isogravimetry albumin clearance eased in proportion to increases in plasma colloid osmotic pressure.

he data suggesting a filtration rate dependent transport of macromolecules from blood tissue will first be discussed since similar findings have been earlier presented in numerous siological studies (*e.g.* Szabo, Magyar and Papp 1963 Appelgren, Jacobsson and Kjellmer 6, Haddy Scott and Grega 1972, Lassen, Parving and Rossing 1974, Friedman 1976, Klein *et al.* 1977 Rippe and Folkow 1977). According to the observations by Grotte (1956)

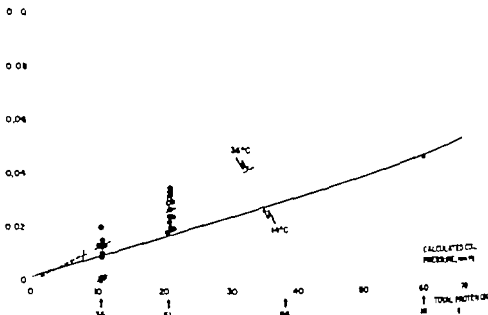


Fig. 3 Initial albumin clearance during isogravimetry plotted against calculated serum colloid osmotic pressure (cf Landis and Pappenheimer 1963) at protein concentrations of 111 g/l, 86 g/l and 61 g/l together with data obtained by extrapolation to isogravimetry of clearance values at various filtration rates for perfusates with 61 g/l and 36 g/l protein concentration The value on β (see Fig. 1 and 2) employed in the extrapolations was 0.072. The Figure shows that the albumin clearance during isogravimetry increases with increasing serum colloid osmotic pressure reflecting an augmented convectional transport of albumin in connection with increases in isogravimetric capillary pressure At a serum colloid osmotic pressure of 8 mmHg (vertical hatched line) transcapillary colloid osmotic pressure difference is zero and therefore isogravimetry is only possible if transcapillary hydrostatic pressure difference were zero. In this theoretical situation albumin transport will occur exclusively by dissipative mechanisms. The dissipative component of tracer transport, which at maximal vasodilatation and at the prevailing flows (≈ 10 ml/min 100 g) should be independent of the level of isogravimetric capillary pressure was estimated to around ... ml/min 100 g.

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3 *Initial albumin clearance at different perfusate protein concentrations at normal temperature and during tissue cooling*

Using perfusates with different contents of serum protein, the initial albumin clearance values were plotted against perfusate colloid osmotic pressures, calculated according to Landis and Pappenheimer (1963) Fig. 3 presents in such a diagram the albumin clearance values during isogravimetry at protein concentrations of 111 g/l, 86 g/l and 61 g/l, together with clearances for perfusates with 61 g/l and 36 g/l protein concentrations during different rates of filtration, from which the isogravimetric values were extrapolated. It is then seen that also during isogravimetry albumin clearance increases with increasing perfusate c...

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and earlier by White, Field and Drinker (1933), on the other hand, macromolecular tra from blood to lymph seemed to be fairly independent of lymph flow rate. In the pr study the initial albumin clearance rose largely in proportion to increases in filtratio in all three experimental groups where this relationship was tested, *i.e.* during tissue perfusate cooling, during perfusion with undiluted horse serum and with diluted h serum at normal temperature. The coefficient of the linear regression of initial alb clearance to filtration rate (β) varied between 0 063 and 0 080 (mean 0.072). Accordi eq 2 (see Theoretical considerations) this would correspond to a mean solvent drag r tion coefficient for albumin (σ_f) of about 0 85–0.86 as deduced from the solvent drag of the general transport expression of Kedem and Katchalsky (1958) They here as that the mean tracer concentration in the large pores is about half that in plasma (C_p $C_p/2$ and $\beta = (1 - \sigma_f)/2$, when the interstitial tracer concentration is near zero.

However the present results indicate that the albumin transport from blood to ti largely *filtration* dependent in which case its concentration in the large pores sho closer to C_p than $C_p/2$) and β closer to $1 - \sigma_f$ than to $(1 - \sigma_f)/2$ (Thomas and Mikulecky This implies a solvent drag reflection coefficient for albumin around 0.93 which is in ment with the osmotic reflection coefficient for plasma proteins obtained by *e.g.* Pa heimer and Soto Rivera (1948) and by Areekul (1969). In the present study it was no sible to determine whether σ_f for albumin is filtration rate dependent, as sugges Brace, Granger and Taylor (1977) on the basis of theoretical considerations on the tw capillary model

Inserting into Eq 2 the value of 0.93 for σ the mean CFC value for this prepara 0 036 ml/min 100 g and a transcapillary colloid osmotic pressure difference of 12.5 (*cf* Fadnes and Aukland 1977) gives an initial albumin clearance value of 0.029 ml/mi g, if diffusion transport is set to zero With a σ value of 0 856 (assuming a mea concentration in the pore of $C_p/2$) approximately the same value on initial albumin cle (0 028 ml/min 100 g) is obtained. This value is in good agreement with the experim obtained 0 0291 ml/min 100 g. However it should again be pointed out that the th cally computed value of 0 028–0 029 gives a maximal estimation of the convection bution to transport, which in reality is likely to be somewhat lower since the term ml/min 100 g must include some contribution by diffusion of albumin, as described

During isogravimetry the passage of tracer albumin from blood to tissue was large portional to perfusate colloid osmotic pressure, in good agreement with Eq (2) and supp the presence of large pore filtration also during isogravimetry (see theoretical consider In case the isogravimetric capillary pressure is elevated by increasing perfusate osmotic pressure, this results in an increased unidirectional protein transport by m bulk flow through the large pores. If however large pore filtration were the only m albumin transfer in the theoretical situation of isogravimetry when perfusate colloid o pressure is lowered enough as to equal that in the interstitial fluid, initial albumin cle would be zero This was not the case, as seen from Fig. 3 because the extrapolated v initial albumin clearance during isogravimetry at a perfusate colloid osmotic pres 8 mmHg was around 0 09–0.10 ml/min 100 g, or about one third of the clearanc at the (for rats) normal perfusate colloid osmotic pressure of around 20 mmHg. Thi

reflects that there is a non-filtrative component of isogravimetric albumin transport perfusion with ordinary plasma at maximal vasodilatation, and essentially all of it theoretical situation seems to represent diffusion of tracer albumin, as discussed below ling the tissues to 13–15°C by reducing perfusate temperature to below 5°C decreased gravimetric albumin clearance to about 60% of control, as seen from Fig. 1 and 3 ary permeability characteristics did, however not change significantly during cooling sured both by CFC measurements, which decreased in due proportion to the tempera-ependent viscosity increase of water i.e. from 0.036 ± 0.001 to 0.024 ± 0.002 ml/min g 100 g, and also by the fact that the solvent drag coefficient (σ_f) for albumin re d about unchanged (Fig. 1). Therefore, the decreases of both CFC and isogravimetric in clearance could be satisfactorily ascribed to the predicted increase in viscosity ac ng to Poiseuille's law. Also diffusion decreases in inverse proportion to the viscosity se and in addition in direct proportion to the decrease in absolute temperature (Stokes-tein relation, cf. Landis and Pappenheimer 1963).

ese cooling data are incompatible with the concept that vesicular transport should ally be a major mechanism for macromolecular transport across capillary walls, as tedly suggested by e.g. Renkin and coworkers (1970, 1974, 1977) and by many morpho-al experts (e.g. Bruns and Palade 1968). Micropinocytosis, a general cell phenomenon nt also in epithelial cells, fibroblasts, smooth muscle and striated muscle, etc. proves very sensitive to cooling (Steinman, Silver and Cohn 1974, Allison and Davies 1974).

n fibroblasts the QT_{10} for micropinocytosis is 2.7 (Steinman, Silver and Cohn 1974) ying that micropinocytotic activity at 14°C should be only about 10% of that at 37°C isogravimetric albumin clearance during cooling to 14°C was, however reduced only bout 60% of that during normal tissue temperature, which closely fits the temperature-ndent reduction of filtration and diffusion capacities.

erefore, the contribution of vesicular transport to transcapillary passage of albumin, if t the whole occurs, must be a largely insignificant part of the total dissipative fraction e albumin clearance, which latter during normal plasma perfusion at isogravimetry unts to about 30% of total clearance. In an earlier study (Rippe and Folkow 1977) ing hardly at all altered the transcapillary labelled albumin clearance at equal levels of ation. The reason is that horse serum diluted to 60% was here used as perfusate and surements were performed at moderate to high filtration rates, 0.4–0.5 ml/min. If the ation rates and albumin clearance values in that study (around 0.03 ml/min 100 g) are apolated to isogravimetry using the corresponding value on σ of 0.06, an isogravimetric rance value near to zero is obtained. Thus, the effects of cooling on isogravimetric min clearance should, in fact, here be quantitatively almost negligible, because isogravi-ric mean capillary pressure is then very low as is also tracer albumin clearance. These umstances explain the insignificant difference between the theoretically parallel regres-lines of albumin clearance vs. filtration rate for this diluted perfusate at normal and low peratures (see above).

might perhaps be argued that vesicular transport in capillary endothelium is insensitive cooling, as suggested on the basis of non-dynamic morphologic data (Jennings and rey 1967, Bankston 1973), and that it is also pressure-sensitive (cf. Johansson 1977)

which would seem to invalidate the conclusions made above. However as mentioned [illegible] dynamic studies of micropinocytosis show that it is, indeed highly sensitive to [illegible] should further be stressed that it is impossible to follow the dynamics of vesicles by purely morphological techniques. For instance, the number of intracellular [illegible] well be increased during cooling even if micropinocytosis has then completely ceased [illegible] case exocytosis is slightly more sensitive to cooling than endocytosis. Also the pos[illegible] of a pressure dependent micropinocytosis seems highly unlikely as this would imply [illegible] capillary vesicular transport is in essence unidirectional causing a marked membrane tr[illegible] from the luminal to the abluminal side of the endothelial cell, as evident from the follo[illegible] calculation About 10 vesicles of 30 nm radius would be transported from the lu[illegible] the abluminal side per minute to account for the measured isogravimetric albumin clear[illegible] of 0 02–0 03 ml/min 100 g. The vesicles needed for such a transport of albumin during [illegible] minute represents a capillary membrane area of 1 m /100 g tissue, which exceeds the [illegible] capillary surface area for that amount of muscle tissue (0.7 m /100 g), according to [illegible] and Pappenheimer (1963) This would imply a tremendous turnover of cell membr[illegible] which should moreover be largely temperature-insensitive, *i.e* independent of cell met[illegible] lism to explain the present data. Furthermore, increases in filtration rate to around [illegible] ml/min 100 g, or a doubling of perfusate colloid osmotic pressure, were found to [illegible] the initial albumin clearance. If ascribed to augmented micropinocytosis, the total [illegible] endothelial membrane would then be transferred to the abluminal side in about 20 [illegible]

Already on the basis of such logistical deductions it is clear that the concept of [illegible] pinocytotic transport of macromolecules across the endothelial cells can hardly be [illegible] and, further the present experimental results provide no evidence for this concept. [illegible] bulk flow by means of filtration through large pores, and to a less extent diffusion [illegible] these pores, constitute the mechanisms for the normal passage of plasma proteins [illegible] interstitial space This passage can be greatly accentuated when substances like hist[illegible] and bradykinin open up 'venular leaks a process that is counterbalanced by circ[illegible] catecholamines via a β-receptor mediated inhibitory influence (Rippe and Grega 1978)

It remains to be seen which are the true functions of endothelial micropinocytosis, [illegible] no doubt reflects some important mechanism. It may for example, rather represent [illegible] portant microphagocytotic functions, associated with *e.g* enzymatic destruction of [illegible] blood-borne substances and capillary plugging material, or/and reflect a slow release [illegible] blood stream and interstitial fluid of functionally important factors produced by the [illegible] thelial cells. It is here interesting that the cerebral capillaries, with very restricted exch[illegible] characteristics, show very little of micropinocytotic vesicles. On the other hand, the l[illegible] capillaries, mainly designed for rapid transfer of oxygen and carbondioxide but, like [illegible] cerebral capillaries, with very limited exchange of water-soluble constituents, show exte[illegible] amounts of micropinocytotic vesicles (Waaler 1971) The endothelium of these lung [illegible] laries may well be responsible for the efficient clearance from the blood steam that [illegible] in the lungs of a number of vasoactive agents like prostaglandins, kinins, and may also [illegible] complish the final elimination of larger particles, which are caught by the 'sieving' func[illegible] of the pulmonary capillary network. It might be clearing functions like these that are [illegible] phologically expressed in terms of the endothelial microvesicles and invagination proce[illegible]

ally though further dealt with in a subsequent study the increased albumin permeabil- occurring first at venous pressures above 40–50 mmHg, also described earlier (e.g. n et al 1932, Halpern, Musso and Neven 1955 Renkin et al. 1977), should be briefly ented here. Such high venous pressures are far beyond the physiological bounds for rats , unlike larger animals are exposed to only small hydrostatic pressure variations upon es in posture. Nevertheless, there was in the present experiments no evidence of any ched pore phenomenon even at capillary pressures up towards 50 mmHg, and certainly t physiologic' capillary pressures, though such stretching has been frequently discussed Wasserman, Loeb and Mayerson (1955) suggested this phenomenon. The exact anisms behind the increased microvascular transfer of proteins at markedly elevated us pressures are not known. Several possibilities must be considered, namely a radius ase affecting the small pores or/and a forced opening of preformed large pores, alternat that more or less irreversible lesional 'rifts occur throughout the microvascular walls Rippe, Kamiya and Folkow 1978). These problems will be explored in detail in experi- ts here also filtration and small molecular diffusion are followed simultaneously This of altered microvascular permeability should be clearly distinguished from that seen ng capillary exposure to high dextran concentrations (e.g. Kaljser Lindedald and Rieger , Arecki 1969 Rippe and Folkow 1977), which at least in part can explain the per bility shifts observed in vivo after dextran infusions (e.g. Parving et al 1974), earlier ibed to the stretched pore phenomenon

To summarize, the present results, obtained during three principally different test ma ures, suggest that the main mechanism behind the normal transmicrovascular transfer of umin is bulk filtration through large pores, even during complete isogravimetry This es a unidirectional vessel to tissue transport of plasma proteins by means of an isogravi- ric 'volume circulation in the capillary membrane, which seems to account for some 70% he albumin transfer and increases in proportion at elevations of isogravimetric capillary ssure. Only around 30% of the albumin transport during isogravimetry at normal plasma oid osmotic pressure seems to take place by dissipative mechanisms, where diffusion ably dominates and a minor fraction, at most, might be due to a true vesicular transport, for this there is no direct evidence. Further such vesicular transport process should be mixed up with the possible formation of large pores by means of vesicular confluence monescu, Simionescu and Palade 1975), which in that case allows macromolecular nsport by filtration and diffusion. Again, there is so far no direct evidence for the forma- of such 'transendothelial' large pores to an extent significant for normal capillary nsfer processes (Wissig and Williams 1978) though the possibility cannot be excluded. It would anyway in that case not imply any fundamentally different situation from the 'classi- ' physiological concept of a small pore—large pore population for diffusion-filtration xchange, as outlined in Introduction.

study was supported by grants from the Swedish Medical Research Council (14X-00016) and from Swedish Faculty of Medicine, University of Goteborg. Thanks are due to Mrs Gertrud Karlsson for led and devoted technical assistance.

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ility though further dealt with in a subsequent study the increased albumin permeability occurring first at venous pressures above 40–50 mmHg, also described earlier (e.g. ... et al. 1932, Halpern, Musso and Neven 1955 Renkin et al. 1977), should be briefly ... ented here. Such high venous pressures are far beyond the physiological bounds for rats ... unlike larger animals are exposed to only small hydrostatic pressure variations upon ... es in posture. Nevertheless, there was in the present experiments no evidence of any ... hed pore phenomenon even at capillary pressures up towards 50 mmHg, and certainly ... 'physiologic' capillary pressures, though such stretching has been frequently discussed ... Wasserman, Loeb and Mayerson (1955) suggested this phenomenon. The exact ... anisms behind the increased microvascular transfer of proteins at markedly elevated ... ry pressures are not known. Several possibilities must be considered, namely a radius ... ge affecting the small pores or/and a forced opening of preformed large pores, alternat... that more or less irreversible lesional 'rifts' occur throughout the microvascular walls (Rippe, Kamiya and Folkow 1978). These problems will be explored in detail in experi... s where also filtration and small molecular diffusion are followed simultaneously. This ... of altered microvascular permeability should be clearly distinguished from that seen ... at capillary exposure to high dextran concentrations (e.g. Kafüser Liljedahl and Rieger ... Arcekul 1969 Rippe and Folkow 1977), which at least in part can explain the per... ability shifts observed in vivo after dextran infusions (e.g. Parving et al. 1974), earlier ... bed to the 'stretched pore phenomenon.

To summarize, the present results, obtained during three principally different test ... ures, suggest that the main mechanism behind the normal transmicrovascular transfer of ... min is bulk filtration through large pores, even during complete isogravimetry. This ... ses unidirectional ... nnel to tissue transport of plasma proteins by means of an isogravi... ric 'volume circulation' in the capillary membrane, which seems to account for some 70 % ... he albumin transfer and increases in proportion at elevations of isogravimetric capillary ... sure. Only around 30 % of the albumin transport during isogravimetry at normal plasma ... oid osmotic pressure seems to take place by dissipative mechanisms, where diffusion ... ably dominates and a minor fraction, at most, might be due to a true vesicular ... for this there is no direct evidence. Further such a vesicular transport process ... be mixed up with the possible formation of large pores by means of vesicular coal... (Simionescu, Simionescu and Palade 1975), which in that case allows macromolecular ... nsport by filtration and diffusion. Again, there is so far no direct evidence for the forma... n of such 'transendothelial' large pores to an extent significant for normal capillary ... nsfer processes (Wissig and Williams 1978) though the possibility cannot be excluded. It ... ould anyway in that case not imply any fundamentally different situation from the classi... physiological concept of small pore—large pore population for diffusion-filtration ... change, as outlined in Introduction.

... study was supported by grants from the Swedish Medical Research Council (14X-00016) and from ... Swedish Faculty of Medicine, University of Göteborg. Thanks are due to Mrs Gertrud Karlsson for ... illed and devoted technical assistance.

which would seem to invalidate the conclusions made above. However as mentioned dynamic studies of micropinocytosis show that it is, indeed, highly sensitive to cooling should further be stressed that it is impossible to follow the dynamics of vesicular transport by purely morphological techniques. For instance, the number of intracellular vesicles may well be increased during cooling even if micropinocytosis has then completely ceased in case exocytosis is slightly more sensitive to cooling than endocytosis. Also the possibility of a pressure dependent micropinocytosis seems highly unlikely as this would imply capillary vesicular transport is in essence unidirectional causing a marked membrane transfer from the luminal to the abluminal side of the endothelial cell as evident from the following calculation About 10^1 vesicles of 30 nm radius would be transported from the luminal to the abluminal side per minute to account for the measured isogravimetric albumin clearance of 0 02–0 03 ml/min 100 g. The vesicles needed for such a transport of albumin during one minute represents a capillary membrane area of 1 m /100 g tissue, which exceeds the capillary surface area for that amount of muscle tissue (0.7 m /100 g), according to Renkin and Pappenheimer (1963) This would imply a tremendous turnover of cell membrane which should, moreover be largely temperature-insensitive, i.e. independent of cell metabolism to explain the present data. Furthermore, increases in filtration rate to around 14 ml/min 100 g, or a doubling of perfusate colloid osmotic pressure, were found to [illegible] the initial albumin clearance. If ascribed to augmented micropinocytosis, the total endothelial membrane would then be transferred to the abluminal side in about 20 s.

Already on the basis of such 'logistical' deductions it is clear that the concept of micropinocytotic transport of macromolecules across the endothelial cells can hardly be [illegible] and further the present experimental results provide no evidence for this concept. Convective bulk flow by means of filtration through large pores, and to a less extent diffusion through these pores, constitute the mechanisms for the normal passage of plasma proteins into the interstitial space. This passage can be greatly accentuated when substances like histamine and bradykinin open up venular leaks a process that is counterbalanced by circulating catecholamines via a β-receptor mediated inhibitory influence (Rippe and Grega 1978)

It remains to be seen which are the true functions of endothelial micropinocytosis, which no doubt reflects some important mechanism. It may for example, rather represent important microphagocytotic functions, associated with e.g. enzymatic destruction of certain blood borne substances and capillary plugging material or/and reflect a slow release into blood stream and interstitial fluid of functionally important factors produced by the endothelial cells. It is here interesting that the cerebral capillaries, with very restricted exchange characteristics, show very little of micropinocytotic vesicles. On the other hand, the lung capillaries, mainly designed for rapid transfer of oxygen and carbondioxide but, like cerebral capillaries with very limited exchange of water soluble constituents, show extensive amounts of micropinocytotic vesicles (Waaler 1971) The endothelium of these lung capillaries may well be responsible for the efficient clearance from the blood steam that occurs in the lungs of a number of vasoactive agents like prostaglandins, kinins, and may also accomplish the final elimination of larger particles, which are caught by the sieving function of the pulmonary capillary network. It might be clearing functions like these that are morphologically expressed in terms of the endothelial microvesicles and invagination processes

V the interstitial fluid volume. Provided that PS is relatively small and the two solvent drag effects has the differential transport are dominant, the mean concentration of the macromolecular tracer pores, $\bar{C}$, may be nearly equal to the plasma concentration, C_p (see Discussion).

$$\bar{C} \approx C_p \qquad \text{Eq (II 2)}$$

the net filtration rate, F_w is maintained constant, the interstitial fluid volume V is

$$V_1 = V + F\ t \qquad \text{Eq (II-3)}$$

net water filtration alone all dilute the interstitial fluid but the protein flux simultaneously tends to interstitial colloid osmolarity here their balance determines the final change in π_p. Since the dated amount of protein must be equal to the tracer amount, $(C_1 V_1)$, multiplied by the protein/ concentration ratio in plasma.

$$\pi_1 = (\ _pV + C_1 V\ \pi_p/C_p)/V = \pi_{p0} V_0/V + \pi_p C_1/C_p \qquad \text{Eq (II-4)}$$

π_{p0} π_1 at time zero.

above reasonable correction between the apparent clearance, CL as measured in the experiments actual clearance CL of the tracer the following calculations were performed.

actual clearance CL is theoretically defined as

$$CL = F_p/C_p \quad \text{at } t = 0 \quad (C_1 \quad 0)$$

rom Eqs (II-1) and (II 2)

$$CL = PS \quad \sigma(1 \quad)CFC\Delta\pi_e + (1 \quad \sigma)F \qquad \text{Eq (II 5)}$$

and clearance at no net filtration, CL_1^0 is given by setting $F = 0$ in Eq (II-5).

$$CL_1 \quad PS \quad \sigma(1-\sigma)CFC\Delta\pi_e \qquad \text{Eq (II-6)}$$

the apparent clearance, CL at time t is given by

$$CL \quad I_p/(tC_p) - C_1 V_1/(tC_p) - \int^t F \ dt/(C_p t)$$

following approximate expression for CL for relatively small PS is obtained by integrating Eq (II 1) ...,

$$CL \quad CL \quad CL_1^0(\pi_p/\Delta \ _e)\left(\int^t C_1\,dt\right) \Big/ (C\ t) + CL_1^0(1 - \pi_p/\Delta\pi_e)\left(\int^t \frac{F\ t}{F\ t+V}\,dt\right) \Big/ t \qquad (\text{II } 7)$$

assuming that

$$t\int^t C\ dt \quad C_1/2 \approx CL \quad C_p/(2V\ t)$$

substituting together with

$$t\int^t \frac{F\ t}{F \quad V}\,dt \quad 1 - \frac{\ln(1+\lambda)}{\lambda} \qquad (\lambda = F\ t/V_0)$$

Eq (II-7), Eq 5 is obtained. Eq 4 can be obtained from Eq 5 by setting $F = 0$ and writing $CL = CL_1$

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Appendix I

According to the theory of irreversible thermodynamics, the volume flows through the small r large pores respectively F_{vs} and F_{vl}, are given by

$$F_{vs} = CFC_s(\Delta P - \sigma_{ds}\Delta\pi)$$

$$F_{vl} = CFC_l(\Delta P - \sigma_{dl}\Delta\pi)$$

where $CFC_{s,l}$ and $\sigma_{ds,l}$ are the filtration coefficients and the colloid osmotic reflection coefficient small and large pores respectively and, ΔP and $\Delta\pi$ are the hydrostatic and osmotic pressure differ between plasma and interstitial fluid. The total net volume flow F is then

$$F_v = F_{vs} + F_{vl}$$

and consequently the total filtration coefficient CFC and the weighted colloid osmotic reflection coefficient for the whole capillary membrane σ_d are:

$$CFC = CFC_s + CFC_l$$

$$\sigma_d = \sigma_{ds}\,CFC_s/CFC + \sigma_{dl}\,CFC_l/CFC$$

Similarly the albumin (tracer) fluxes through the small and large pores F_{as} and F_{al} are

$$F_{as} = PS_s\Delta C + (1-\sigma_{fs})F_{vs}\overline{C}_s$$

$$F_{al} = PS_l\Delta C + (1-\sigma_{fl})F_{vl}\overline{C}_l$$

where $PS_{s,l}$ and $\sigma_{fs,l}$ are the permeability coefficient respective the solvent drag reflection coefficient for albumin in the small and large pores, ΔC the albumin (tracer) concentration difference between plasma and interstitium and $\overline{C}_{s,l}$ the mean albumin concentration in the small and large pores respectively.

Assuming that the serum proteins freely pass through the large pores but do not pass through the small pores, it follows that,

$$\sigma_{ds} \approx \sigma_{fs} = 1 \quad \sigma_{dl} \approx \sigma_{fl} = 0 \quad 1-\sigma_d = \frac{CFC_l}{CFC} \quad P_{as} = 0 \quad F_{as} = 0 \quad \text{and} \quad P_{al} = P$$

where PS is the weighted albumin (tracer) permeability for the capillary membrane as a whole. Then the total solute flux F is,

$$F = F_{al} = PS_l\Delta C + F_{vl}\overline{C}_l = PS\Delta C + CFC(1-\sigma_d)\Delta P\overline{C}_l$$

Further from Eq (I), I

$$F = CFC(\Delta P - \sigma_d\Delta\pi)$$

we obtain

$$\Delta P = \frac{F + \sigma_d CFC\Delta\pi}{CFC}$$

and substituting Eq (I 10) into Eq (I 9),

$$F = PS\Delta C + \sigma_d(1-\sigma_d)CFC\overline{C}_l\Delta\pi + (1-\sigma_d)\overline{C}_l F$$

Thus, Eq (2) in the text is obtained by rewriting σ_d to σ and $\overline{C}_l$ to $\overline{C}$. (For more generalized deriv see Horne, 1974.) Notice that in Eq (I 11), $\Delta\pi$ and σ_d represent the colloid osmotic pressure diff and the weighted osmotic reflection coefficient for all the serum proteins while ΔC and $\overline{C}_l$ represent transcapillary concentration difference respective the mean large pore concentration of albumin

Appendix II (A. Kamiya)

From Eq 2 (see theoretical considerations) the transient changes in the interstitial tracer concentration C_i is given by

$$\frac{d(C_i V_i)}{dt} = F = PS(C_p - C_i) + \sigma(1-\sigma)\,CFC\Delta\pi\overline{C} + (1-\sigma)F\,\overline{C}$$

the interstitial fluid volume. Provided that PS is relatively small and the two solvent drag effects on the differential transport are dominant, the mean concentration of the macromolecular tracer [illegible] pores, $\bar{C}$, may be nearly equal to the plasma concentration, C_p (see *Discussion*).

$$\bar{C} = C_p \qquad \text{Eq (II-2)}$$

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$$CL \quad PS + \sigma(1 \quad \sigma) CPC\Delta\pi_p + (1 - \sigma) F \qquad \text{Eq (II-5)}$$

[illegible] clearance at no net filtration, CL_1 is given by setting $F = 0$ in Eq (II 5).

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The apparent clearance, CL at time is given by

$$CL = f_t/(tC_p) \quad C_t V_t/(tC_p) = \int^t F \; dt/(C_p\, t)$$

[illegible] approximate expression for CL for relatively small PS is obtained by integrating Eq (II-1) [illegible],

$$CL \quad CL \quad CL_1^0(\pi_p/\Delta\pi_p)\left(\int^t C_t\, dt\right) \Big/ (C_p\, t) + CL_1^0(1 - \pi_p/\Delta\pi_p)\left(\int^t \frac{F\,t}{F\,t + V}\, dt\right) \Big/ t \qquad \text{(II-7)}$$

[illegible] that

$$\frac{1}{t}\int^t C_t\, dt \quad C_t/2 = CL \quad C_p/(2V\,0)$$

[illegible] together with

$$\frac{1}{t}\int^t \frac{F\,t}{F} \frac{}{V}\, dt \quad 1 - \frac{\ln(1+\lambda)}{\lambda} \qquad (\lambda \quad F\,t/V_0)$$

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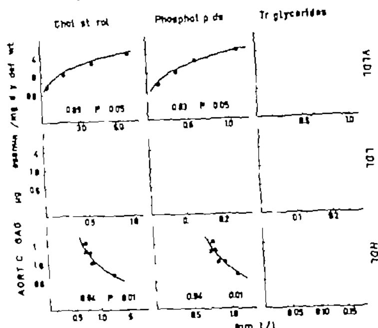
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...lationships between the concentration of aortic glycosaminoglycans (GAGs) and constituent plasma very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density ...ins (HDL) in rats fed the high cholesterol, olive oil diet for 0–3 ...eeks.

...present biochemical study was carried out to detect early effects of a high cholesterol, ...l diet on the intima-media of rat aorta by measuring its concentrations of cholesterol, ...osaminoglycans (GAGs) and collagen. The simultaneous changes in plasma lipoprotein during a three weeks induction period of hypercholesterolemia were recorded to find ...le correlations between the concentrations of lipids in plasma lipoprotein fractions and ...l connective tissue components.

Materials and methods

...prague-Dawley and Wistar rats, aged 10–14 weeks, were used. Control rats were kept on standard ...tory diet (Hankkija Oy, Helsinki, Finland, cholesterol concentration 0.4 g/kg, 10 ... of total ...fat, 38 ... carbohydrate, 38 ... protein). Experimental rats were fed ad libitum on ... high cholesterol, ...l diet (Saarto and Pälsse 1965: cholesterol concentration 15 g/kg, olive oil 200 g/kg, sucrose 520 g/kg, ...-cholate 5 g/kg, 38 ... of total calories fat, 45% carbohydrate, 17% protein). This diet does not ... the weight gain of the rats (Pelkonen 1973). It elevates the concentration of plasma cholesterol to ...mol/l in 3 weeks with no further increase in 7 weeks, whereas plasma triglyceride concentration is only ...y affected by the diet (Pelkonen 1973, Pelkonen et al. 1976).

...*erimental* In experiment A rats (... 18) were fed ... standard laboratory diet or the high cholesterol, ...oil diet for one week or 22 weeks. Four-five rats from both dietary groups were killed at one week ... 22 weeks.

...periment B rats (n ... 37) were fed the high cholesterol, olive oil diet for 0–3 weeks. After 0, 1, 3, 7 ...(21) days on the diet 4–5 rats were killed. 8 rats were refed the standard laboratory diet, 4 of these rats ...killed both after 1 and 3 weeks on the standard diet.

...*paration of the aortas.* Rats were killed by open heart puncture under ether anesthesia after an over... fast. The descending thoracic aorta (length 25–35 mm) was dissected and stored at −25°C until ...

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Rapid increase of glycosaminoglycans in the aorta of hypercholesterolemic rats, a negative correlation with plasma HDL concentration

By

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Abstract

TAMMI M., T RÖNNEMAA and J VIIKARI *Rapid increase of glycosaminoglycans in the aorta of hypercholesterolemic rats a negative correlation with plasma HDL concentration* Acta physiol scand 1979 *105* 188–194

Rats were kept either on a standard laboratory diet or a high cholesterol, olive oil diet for periods from 1 day to 22 weeks. The effect of the high cholesterol, olive oil diet on the concentrations of the glycosaminoglycans (GAGs) and collagen in aortic intima-media, were studied and the developing lipidemia was characterized. The concentration of cholesterol in rat aorta was increased after [illegible] high cholesterol olive oil diet while collagen concentration was not affected. On the contrary concentration of aortic sulphated GAG was significantly increased already after one week [illegible] cholesterol, olive oil diet The diet increased the formation of a cholesterol-rich very low density lipo[protein] (VLDL), decreased high density lipoprotein (HDL)-associated cholesterol and phospholipids, [but had] virtually no effect on low density lipoprotein (LDL)-lipids. The concentrations of VLDL-cholesterol [and] phospholipids showed positive correlations with the concentration of aortic GAGs (r [illegible] respectively $P<0.05$ for both) Stronger (negative) correlations were found between aortic GAG [and] HDL-cholesterol and -phospholipids ($r = -0.94$ for both, $P<0.01$) suggesting that HDL may [be] in the control of arterial sulphated GAG concentration.

Key words: Aorta cholesterol collagen glycosaminoglycans, sulphated hyaluronic acid hypercholesterolemia lipoproteins, high density lipoproteins, low density lipoproteins, very low density [lipoproteins]

Epidemiological studies have demonstrated that elevated concentrations of plasma [low] density lipoproteins (LDL) and very low density lipoproteins (VLDL) are associated with a high risk of atherosclerosis and coronary heart disease (Carlson and Ericsson 1975) On the other hand, a strong correlation has been reported between decreased concentration of high density lipoproteins (HDL) and coronary heart disease (Barr [...] Nikkilä 1953 Miller and Miller 1975 Gordon et al. 1977) It is thus possible that the plasma lipoprotein fractions have different influences on arterial connective [tissue] metabolism, which is known to be affected during the early phase of atherogenesis ([...] 1960 Ross and Glomset 1973)

II. Concentration of aortic glycosaminoglycans (GAGs, μg hexosamine/mg dry defatted weight) and constituent lipids (mmol/l) in plasma very low density (VLDL), low density (LDL) and high density lipoproteins (HDL) in rats fed the high cholesterol, olive oil diet.

	Days on high cholesterol, olive oil diet						Days back on standard diet	
	0	1	3	7	14	21	7	21
GAGs	0.75±0.10	1.02	1.26	1.24	1.04	1.44	0.96	0.87
cholesterol	0.46±0.07	1.80	1.63	2.76	2.55	6.87	1.10	0.59
phospholipid	0.15±0.02	0.37	0.28	0.61	0.48	1.14	0.35	0.23
triglyceride	0.41±0.12	0.93	0.70	0.92	0.73	0.60	0.61	0.55
cholesterol	0.85±0.13	0.54	0.40	0.70	0.55	0.84	0.33	0.51
phospholipid	0.23±0.04	0.19	0.20	0.22	0.14	0.22	0.19	0.16
triglyceride	0.18±0.04	0.10	0.14	0.23	0.13	0.10	0.14	0.21
cholesterol	1.28±0.17	0.86	0.74	0.83	0.68	0.75	1.71	1.56
phospholipid	1.07±0.05	0.92	0.80	0.74	0.84	0.77	1.19	0.96
triglyceride	0.15±0.02	0.06	0.08	0.15	0.09	0.11	0.16	0.11

[Values] for each day represent the mean of 4–5 rats, analysed individually at day 0 (±S.E.) and in pools [at] later time points.

[...]ted. The experimental diet did not significantly affect the defatted dry weight of the [...], when calculated per intimal surface area (Table I). A slight increase in aortic [chole]sterol concentration was found at 22 weeks, while the concentration of collagen was [un]changed (Table I). In contrast to the slow increase in aortic cholesterol the concentra[tion] of total GAGs was increased already after one week on the high cholesterol, olive oil [diet] (Table I). This appeared to be due to an increase in sulphated GAGs since the con[centra]tion of hyaluronic acid was not significantly affected by the diet.

[In] Experiment B the aortic GAG-concentration increased within a few days on the high [chol]esterol, olive oil diet (Table II). Plasma VLDL-cholesterol and -phospholipids increased [up t]o 3 weeks, while LDL-lipids were not affected by the diet (Table II). Plasma HDL-[chol]esterol and -phospholipids decreased immediately after the onset of the diet. The [incr]eased aortic GAG-concentration decreased back to the initial level after restoration of [the] standard diet. The changes in VLDL and HDL were also rapidly reversible (Table II). [S]tatistically significant correlations were found during the three weeks experiment between [aorti]c GAG-concentration and plasma VLDL-cholesterol and -phospholipids (r = 0.89 and [0.9]4, respectively, P [<] 0.05 for both) and HDL-cholesterol and -phospholipids (r [=] −0.94 [for] both, P [<] 0.01) (Fig. 1). The equations for the curves drawn for VLDL-cholesterol, [VLD]L-phospholipids, HDL-cholesterol and HDL-phospholipids are y [=] 0.94 x[...] y [...] x[...] y [=] 0.95 [...] and y [=] 0.85 x[...] respectively.

Discussion

[The] present lipid diet causes a rapid increase in the concentration of GAGs in rat aorta. [This] early reaction seems to be rather specific to sulphated GAGs because the concentra[tion] of hyaluronic acid, total cholesterol and collagen in the aorta were not significantly

TABLE I Effect of the high cholesterol, olive oil diet on the intima-media of rat aorta. The values, the means ± S.E. of the number of aortas indicated in parenthesis. The dry defatted w- the tissue is e pressed as mg/cm intimal surface area and the other parameters as g defatted weight

Parameter	1 week		22 weeks	
	Experimental (5)	Control (5)	Experimental (4)	Contr (4)
Dry defatted weight	3.98 ± 0 04	3.82 ± 0.11	4.33 ± 0.21	[illegible]
Collagen	243 ± 8	245 ± 10	262 ± 5	[illegible]
Cholesterol	4 64 ± 0.21	5.38 ± 0 11	6.76 ± 0.98	[illegible]
Total GAGs (uronic acid)	2.05 ± 0.10	1 62 ± 0 16	1.90 ± 0.29	[illegible]
Total GAGs (he osamine)	1 69 ± 0.06	1 49 ± 0 09	1 72 ± 0.23	[illegible]
Hyaluronic acid (hexosamine)	0.59 ± 0.04	0.63 ± 0.04	0 56 ± 0 07	[illegible]
Sulphated GAGs (hexosamine)	1 10 ± 0.06	0.86 ± 0 06	1 16 ± 0 17*	0.7[illegible]

* P < 0.05 control aortas as the reference

analyzed The aorta was opened longitudinally and the intima media was stripped off under t[illegible] microscope The length and mea width of the rectangular piece of intima-media tissue then [illegible] estimated with 0.25 mm accuracy for calculation of the intimal surface area.

Determinatio of plasma and aortic lipids. Lipids were extracted from EDTA-plasma ch[illegible] methanol (1 1 /v Folch et al 1957) purified (Svennerholm 1968) and aliquots ere taken for [illegible] mination of cholesterol (Badzio nd Bocron 1966), neutral glycerides (Carlson 1963) and [illegible] (Bartlett 1959 Svanborg and Svennerholm 1961).

The analysis of different plasma lipoprotein fractions was performed accord ng to Viikari [illegible] (1978).

The intima-media samples were rinsed with cold saline cut into small pieces and extracted [illegible] form-methanol (2 1 /v) for 24 h with vigorous shaking and the e traction as repeated [illegible] combined extracts were used f r the determination of total cholesterol.

Determination of collagen and gl cosaminoglycans. After lipid e traction the aortic intima-med[illegible] were lyophilized and their dry weight determined. The samples were digested with papain (Thunell[illegible] duplicate aliquots from the resulting suspension were hydrolyzed in 6 N HCl at 130°C for 3 h[illegible] evaporation f HCl the concentration of hydroxyproline was determined (Woessner 1961) to [illegible] approximat collagen content (Pikkarainen 1968).

The rest f the suspension was centrifuged nd then GAGs were precipitated from the supern[illegible] 1% CPC (cetylpyridinium chloride) in 0.02 M NaCl nd purified by ethanol precipitation (Th[illegible] The ethanol precipitat was dissolved in water and used for the determination of hexosamines ([illegible] uronic acids (Blumenkrantz d Asboe-Hansen 1973) or fractionated into hyaluronic acid and [illegible] GAGs by precipitating sulphated GAGs with 1% CPC in 0.3 M NaCl.

Correlation analysis and statistical calculations. I rder to relate the changes found in the conc[illegible] of plasma lipoprotei lipids on one hand and in aortic GAG on the other regression curves of [illegible] rithmic, exponential and power functions were calculated with a T xas TI 58 calculator. T[illegible] correlation coefficients were btained by using power functions (Fig. 1). Student's t-test was used [illegible] mine the statistical significances.

Results

In Experiment A the plasma lipid values of rats which had been kept for 22 week[illegible] high cholesterol, olive oil diet (cholesterol 7 86 ± 0 9 vs. 1 15 ± 0 07 P < 0.001 p[illegible] lipids 1.52 ± 0.15 vs. 0 71 ± 0.03 P < 0 01 and triglycerides 0.33 ± 0.07 vs. 0.2[illegible] P N.S. values of experimental vs control rats) were at the same level as they are k[illegible] be at 3 and 7 weeks (Pellinkemi 1973) No macroscopic signs of atheroscl[illegible]

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increased (Table I) There were no signs of macroscopic atherosclerosis detectable ev[illegible] longer feeding periods. This is in agreement with earlier studies (Renaud 1974) acc[illegible] to which olive oil in combination with cholesterol, sucrose and cholic acid is rather [illegible] atherogenic in the rat. We consider that the early change in GAGs due to the lipid [illegible] reflects some kind of homeostatic regulation rather than a pathological accumulation [illegible] is supported by the rapid reversibility of the increase in GAGs, when the standard la[illegible]tory diet was restored However accumulation of metachromatic substances in sub[illegible] (Tucker 1971) and increased incorporation of [35]SO into aortic intima-media (Ihers [illegible] are typical signs of developing atherosclerosis in the rat Vijayakumar *et al.* (1975) [illegible] reported that an atherogenic diet in the rat causes first an increase in aortic sul[illegible] GAGs and later with progressive lipid infiltration a decrease in their concentra[illegible] thus seems that a long term feeding of the possibly more atherogenic diets (Ichida [illegible] Kalant 1968, Seethanathan and Kurup 1971) decrease the concentration of aortic sul[illegible] GAGs while a shorter feeding period (Vijayakumar *et al* 1975) and, as in the present [illegible] less atherogenic diet (Table I) increases aortic sulphated GAGs. Therefore, incr[illegible] concentration of sulphated GAGs in the arterial wall is not necessarily an essential fe[illegible] of an already established atherosclerotic lesion but perhaps makes the still normal [illegible] more susceptible to the disease (Mancini *et al* 1965)

In a recent paper Ehrlich and Murray (1978) found that the synthesis of sulphated pr[illegible] glycans by cultured monkey and rabbit aortic smooth muscle cells was stimulated b[illegible] creasing the concentration of human LDL in the growth medium. On the other hand [illegible] preliminary data demonstrate that human sera with high concentrations of HDL [illegible] the synthesis of sulphated GAGs by cultured human aortic smooth muscle cells [illegible] published) The present study suggests that an increase in plasma VLDL and a decrea[illegible] HDL due to the high cholesterol olive oil diet has similar effects on the concentra[illegible] aortic sulphated GAGs *in vivo* In previous papers (Ichida and Kalant 1968, Seethan[illegible] and Kurup 1971 Vijayakumar *et al* 1975) the possible correlations between the conc[illegible]tions of aortic GAGs and the lipids in various lipoprotein fractions have not been deter[illegible] However in an *in vivo* experiment it is difficult to state definitely whether the changes [illegible] concentrations of various lipoprotein fractions, and which of them, cause the increase [illegible]terial GAGs. In any case, our results suggest that among the various lipoprotein frac[illegible] the concentration of HDL is the best predictor for the early increase in the ar[illegible] GAGs.

There are many possibilities how arterial GAGs may be involved in the initia[illegible] atherosclerosis (Kunz 1975 Robinson *et al* 1975) The ability of certain sulphated GAG[illegible] bind and precipitate LDL and VLDL may lead to the accumulation of cholesterol in [illegible] arterial wall with subsequent atherosclerosis (Iverius 1972, Srinivasan *et al* 1972, Holz[illegible] 1976) A high concentration of HDL might inhibit the harmful precipitation by decre[illegible] the amount of sulphated GAGs This could explain the protective effect of high HD[illegible] concentration against atherosclerosis (Glueck *et al* 1975 Gordon *et al* 1977).

The present work was supported by the University Foundation Turku, Finland, and by the Research Science Foundation of Lääke Oy Turku, Finland.

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ECG-changes in the fetal lamb during asphyxia in relation to beta-adrenoceptor stimulation and blockade

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Abstract

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[illegible] changes in the S-T interval of the fetal electrocardiogram (FECG) were studied in 14 lamb [illegible], acutely exteriorized and subjected to graded hypoxia. The aims of the study were to investigate [illegible] beta-adrenoceptor stimulation and hypoxia exerted additive or potentiating effects on the [illegible] and several cardiovascular parameters and whether the hypoxic changes of the FECG could be [illegible] by beta-adrenoceptor blocking agents. The FECG changes were studied in order to correlate them [illegible] cardiovascular function, as measured by heart rate, mean arterial pressure, end diastolic pressure, [illegible] dP/dt and combined cardiac output, estimated by the thermodilution method, as well as with [illegible], acid base status, blood lactate and glucose. Injections of small doses (0.02 to 0.4 $\mu g\ kg^{-1}\ min^{-1}$) [illegible] mimicked the same pattern of changes in the FECG as we have previously recorded during [illegible]. By increasing the isoprenaline dose an increase in the duration of the FECG changes and [illegible] of the T-wave changes was obtained. Propranolol was found to completely abolish the FECG [illegible] induced by isoprenaline, as well as by mild hypoxia. During severe hypoxia the FECG changes [illegible] be abolished by propranolol. Our previous findings indicated that the hypoxic changes could be [illegible] as a sign of myocardial glycolysis. Thus, the present finding that even small doses of isoprenaline [illegible] to the fetus, initiates the same pattern of FECG changes corroborates this hypothesis.

[illegible] fetal electrocardiogram (FECG) is used in clinical practice mainly as a means to obtain [illegible] of fetal heart rate (FHR). While it is generally accepted that FHR is an [illegible] of fetal well-being during pregnancy and labour the diagnostic value of the [illegible]G is controversial. Although alterations in the pattern of the FECG have been detected [illegible] labour (Davidsen 1971, Pardi *et al.* 1974) by using the fetal scalp electrode, these [illegible] are believed to be preceded by changes in the FHR (Hon and Lee 1963, Lee and [illegible] 1965, Davidsen 1971).

[illegible] investigators have shown changes in the FECG during hypoxia using experimental [illegible] (Enhörning and Westin 1954, Stern *et al.* 1961, Gelli and Gyulai 1969, Mueller-[illegible] *et al.* 1971, Myers 1972, Morishima *et al.* 1975). Although changes in the S-T [illegible] were readily apparent, it was stated that the use of FECG for the detection of fetal [illegible] will be limited.

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ECG-changes in the fetal lamb during asphyxia in relation to beta-adrenoceptor stimulation and blockade

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.wave changes in the S–T interval of the fetal electrocardiogram (FECG) were studied in 14 lamb .acutely exteriorized and subjected to graded hypoxia. The aims of the study were to investigate .er beta-adrenoceptor stimulation and hypoxia exerted additive or potentiating effects on the .and several cardiovascular parameters and whether the hypoxic changes of the FECG could be .d by beta-adrenoceptor blocking agents. The FECG changes were studied in order to correlate them .ardiovascular function, as measured by heart rate, mean arterial pressure, end diastolic pressure, .um dP/dt and combined cardiac output, estimated by the thermodilution method, as well as with .tion, acid base status, blood lactate and glucose. Injections of small doses (0.02 to 0.4 μg kg⁻¹ min⁻¹) .prenaline induced the same pattern of changes in the FECG as we have previously recorded during .a. By increasing the isoprenaline dose an increase in the duration of the FECG changes and .tude of the T-wave changes was obtained. Propranolol was found to completely abolish the FECG .es induced by isoprenaline, as well as by mild hypoxia. During severe hypoxia the FECG changes .not be abolished by propranolol. Our previous findings indicated that the hypoxic changes could be .ted as signs of myocardial glycolysis. Thus, the present finding that even small doses of isoprenaline .to the fetus, imitates the same pattern of FECG changes corroborate this hypothesis.

.fetal electrocardiogram (FECG) is used in clinical practice mainly as a means to obtain .ndicator of fetal heart rate (FHR). While it is generally accepted that FHR is an .ator of fetal well-being during pregnancy and labour the diagnostic value of the .G is controversial. Although alterations in the pattern of the FECG have been detected .ng labour (Davidsen 1971, Pardi *et al.* 1974) by using the fetal scalp electrode, these .ations are believed to be preceded by changes in the FHR (Hon and Lee 1963, Lee and . 1965, Davidsen 1971).

.everal investigators have shown changes in the FECG during hypoxia using experimental .als (Enhorning and Westin 1954, Stern *et al.* 1961, Gelli and Gyulai 1969, Mueller .rbach *et al.* 1971, Myers 1972, Morishima *et al.* 1975). Although changes in the S–T .rval were readily apparent, it was stated that the use of FECG for the detection of fetal .hyxia will be limited.

Our own animal experiments revealed progressive and reproducible changes in the S interval of the FECG during graded hypoxia both in mature guinea-pig fetuses and lamb fetuses (Rosén and Kjellmer 1975). The metabolic background of these changes of FECG was studied from different aspects. Rosén and Isaksson (1976) demonstrated parallelity between the FECG changes and the depletion of myocardial glycogen during graded hypoxia in guinea pig fetuses. The significance of this finding was supported by the relationship found between the increase in the amplitude of the T wave and the degree of metabolic acidosis as well as the accumulation of lactate in the exteriorized fetal lamb (Rosén *et al* 1976) These findings together with the relationship found between the appearance of the FECG-changes and decrease in PaO (Rosén, Hökegård and Kjellmer) indicate that the progressive FECG changes reflect myocardial glycogenolysis. Alterations in the FECG pattern consistently preceded signs of failing cardiovascular function and occurred well in advance of any bradycardia (Rosén, Hökegård and Kjellmer 1976).

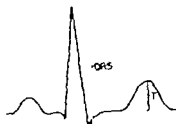
Thus, there are a good many observations indicating that progressive changes in the S interval reflect myocardial glycogenolysis and early hypoxic stress. The augmentation of glycogenolysis in heart muscle by beta-receptor stimulation is well known (Mayer et al 1967) Our own preliminary experiments indicated that the betareceptor stimulating agent isoprenaline, initiated the same typical pattern of changes in the FECG as hypoxia. Therefore it was considered of interest to elucidate the influence of beta-adrenergic stimulation and blockade on the FECG

The aims of the present study were to investigate whether beta-adrenoceptor stimulation and hypoxia exerted additive or potentiating effects in the FECG and several cardiovascular parameters and whether the hypoxic changes of the FECG could be blocked by beta-adrenoceptor blocking agents.

Material and methods

The experiments were conducted on 9 ewes of mixed breed with 14 fetuses acutely exteriorized. The gestational age was dated in 2 ewes and was estimated from fetal weight and crown-rump length in 7 using standard curves (Joubert 1956). I *first series* of 5 ewes with 8 fetuses, the experiments were conducted according to the methods described earlier (Rosén, Hökegård and Kjellmer 1976) Their gestational age ranged from 109 to 132 days (term 147 days) and their weight from 1 060 to 3 370 g (2 ... mean value ± S.E.). The left jugular vein f the fetus was cannulated and 0.1 to 10.0 μg (0 04 to 9 ...) isoprenaline was given v in si gle injections. Via the same catheter the beta-blocking agent, propranolol was given in doses varying from 0.05 to 2 mg (0.02 t 0.83 mg/kg). Fetal nd maternal blood pressure, pH and blood gas tensions were measured, fetal heart rate and end diastolic pressure ... combined fetal cardiac output was estimated d the FECG recorded with CR-lead. The ewes (and thereby the fetuses) were exposed t alternate periods of normoxia and hypoxia. The hypoxia was induced by ventilating the ewe with gas mixtures containing 9–15% O_2 in N_2 and in some cases with an addition of 10–20% CO The hypoxic changes in the ECG pattern were quantified according to the scoring system previously described

Grade I The appearance of negative T wave changes, the amplitude exceeding that of the P wave.
Grade II Maximally negative T wave changes.
Grade III A gradual decrease in the amplitude of the negative T wave changes.
Grade IV An elevation of the S–T segment and the T wave the amplitude of the T wave being higher than that of the P wave.
Grade V A maximal increase in the amplitude of the T wave.
Grade VI A decrease in the amplitude of the T wave during continuo poxia.



Example of the calculation of the T/QRS ratio.

...cond series of 4 ewes with 6 fetuses was used to establish a quantitative dose-response relationship ...en isoprenaline and FECG changes. Their gestational age ranged from 128 to 138 days (132 ± 2, mean ... S.E.) and their weight from 2 330 to 2 950 g (2 530 ± 100). Maternal and fetal blood pressure, blood gases and pH, fetal heart rate and FECG were registered in the same way as in the first series. ...er to get quantitative measurement of the high and peaked T waves, ratio between the amplitude ... QRS complex and the T wave (T/QRS ratio) was calculated (Fig. 1).

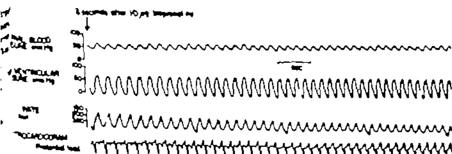
...t ventricular pressure was monitored with micro-tip sensitive catheter (Millar instruments), in... via the left carotid artery. Maximum dP/dt was derived electronically from this signal and con...ously recorded. The thermodilution method (Hedvall et al. 1973) was also used for estimation of the ...ined fetal cardiac output ("CO"), but instead of injecting saline of room temperature into the left ...cle as in the first series, it was injected into the inferior vena cava through a catheter inserted in the ...al vein. The left jugular vein was cannulated and isoprenaline was administered continuously with an ...ion pump in stepwise higher doses of 0.044, 0.076, 0.143 0.22, 0.78 and 4.4 µg/min in order to ...ain dose-response curve. This was done both during normoxia and hypoxia before and after beta-...ceptor block. The beta-blocking agent propranolol was given through the same catheter in the ...gular vein at doses varying from 0.02 to 0.5 mg (0.01 to 0.19 mg/kg).

Results

...adrenoceptor stimulation

...e first series, isoprenaline was given i.v. in single doses ranging from 0.04 to 9.0 µg/kg ... fetuses. In each case isoprenaline induced the same alterations of the FECG pattern as ...xia.

...relationship was found between the dose of isoprenaline and the increase in T wave ...itude, as well as the duration of the ECG changes. Fig. 2 gives an example of ...rdings made after the injection of 1.0 µg (0.4 µg/kg) isoprenaline. The whole sequence ...lterations of the S-T interval, accounted for in the ECG-scoring system is demonstrated.



2. Fetal electrocardiogram changes after the injection of 1.0 µg (0.4 µg/kg) isoprenaline. Note the rapid ... at the S-T interval.

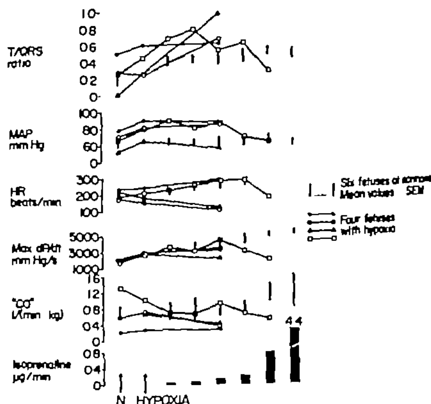


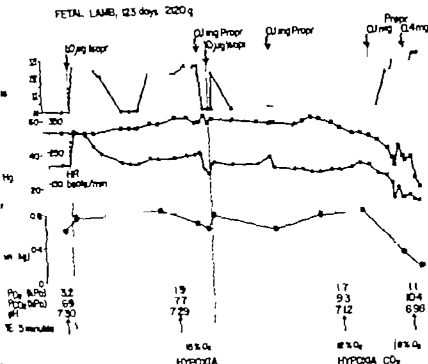
Fig. 3 Effect of isoprenaline infusion on the T/QRS ratio of the fetal electrocardiogram, fetal heart mean arterial pressure maximum dP/dt and combined cardiac output both during normoxia and hy

In order to further elucidate the influence of beta-adrenoceptor stimulation on the FH and cardiovascular function and to estimate a dose response curve, isoprenaline continuously infused in step-wise higher doses in a second series. This group of 6 fe was quite homogeneous regarding gestational age and fetal weight.

ECG-changes Fig. 3 demonstrates the relationship between the increase in T w amplitude and the dose of isoprenaline (6 fetuses) Hypoxia (4 fetuses) had an add effect on the ECG changes which appeared earlier and with a higher T/QRS ratio (Gr V in the scoring system) At the highest dose of isoprenaline given during hypox T/QRS ratio decreased again (Grade VI in the scoring system) in the only animal stu

Cardiovascular function Fig. 3 also demonstrates the effect of isoprenaline on mean art pressure, fetal heart rate, maximum dP/dt and combined cardiac output. Both FH dP/dt and cardiac output increase gradually with increasing doses of isoprenaline. M shows no significant changes. The augmentation of cardiac performance caused by i prenaline infusion was inhibited by the combination of hypoxia and acidosis. Fig. 3 dem strates the combined effect of hypoxia and isoprenaline in 4 fetuses. After an initial associated with hypoxia both MAP FHR, maximum dP/dt and "CO" decrease at incre doses of isoprenaline.

Blood gases, pH lactate and glycose There was no change in PaO during the isopren infusions at normoxia. PaO was 3 19 ± 0.29 kPa before the infusions started and 3.09 ±



4 Changes in the fetal electrocardiogram, graded according to the scoring system, mean arterial ..., fetal heart rate and combined cardiac output after isoprenaline and propranolol injections made during normoxia, hypoxia and the combination of hypoxia and hypercarbia.

he end of the infusions (mean value ± S.E.). pH, however decreased from 7.27 ± 0.04 ... 17 _0 06 and there was a lactate accumulation from 2.85 ± 0.47 to 4 77 ± 1.69 mmol/l. ...d glycose values did not change (1 47 ± 0.28 and 1.53 ± 0.30 mmol/l respectively).

... the 4 fetuses subjected to both hypoxia and isoprenaline infusion the mean PaO... was 3.53 (range 3 19–3.86) before hypoxia and decreased to 1.86 (range 1.06–2.66) ...ing hypoxia and 1.83 (range 1 06–2.52) when isoprenaline infusion was added. The mean ... value was 7 15 (range 6 99–7.24) before hypoxia and decreased to 7.04 (range 6.89–7 18) ...ing hypoxia and isoprenaline infusion. The mean lactate value was 5.94 (range 1 71–...32) before the hypoxic period and the concentration rose to 8.58 (range 1 71 13 16) ...mol/l during hypoxia and isoprenaline infusion. Blood glycose values increased slightly ...m 1.96 (range 1 74–2.20) to 2.20 (range 2.07 2.36) mmol/l. In all these 4 fetuses the ...iod of hypoxia combined with isoprenaline was preceded by periods of isoprenaline ...sion ...thout hypoxia.

...a-adrenoceptor blockade

...opranolol was also given to the 8 fetuses of the first series in order to test whether beta-...renoceptor blockade interfered with the ECG changes induced by hypoxia. The degree of

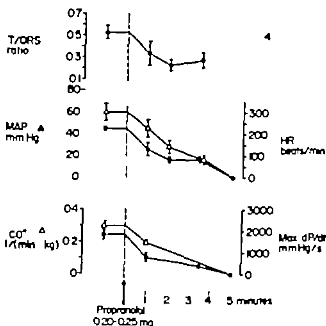


Fig. 5. Effect of propranolol in ... subjected to hypoxia and acidosis ... was 2,21 ± 0.24 kPa before the ... and 1.97 ± 0.39 after the injection ... 6.98 ± 0.13 before the injection and ... 0.09 after the injection (mean ... S E.).

beta blockade was tested by repeated injections of isoprenaline. The ECG changes ... by isoprenaline completely disappeared about 10 s after the propranolol injection.

Fig. 4 demonstrates the pattern of events recorded in a lamb fetus (gestational ... 123 days). The first part of the figure illustrates the effect of isoprenaline (0.47 μg/kg) ... combined cardiac output, heart rate and mean arterial pressure as well as the FE... Alterations in the FECG pattern, elevation of the S–T segment with high and peak... waves, were associated with a tachycardia and an increase in cardiac output from ... 0 78 l/(min kg). A moderate hypoxia, induced by ventilating the ewe with 15% O_2, ... the same pattern of events in the FECG. These changes were immediately block... propranolol (0 05 mg/kg). This dose of propranolol was found to almost completely ... the effect of a large dose of isoprenaline (4 7 μg/kg). In the last part of the figure the ... of beta blockade associated with a severe hypoxia are demonstrated. No FECG ch... were recorded until there were signs of falling cardiovascular function. These FECG ch... could apparently not be blocked even by large doses of propranolol. Propranolol bl... the FECG changes elicited by moderate hypoxia in the two fetuses studied but did not ... the FECG changes associated with a severe hypoxia and a falling circulation (6 cases). T... fetus was extremely susceptible to the combination of hypoxia and propranolol.

In the second series propranolol blocked the cardiovascular effects of isoprenaline in ... same way. These fetuses were also extremely susceptible to the combination of propra... and hypoxia. In 4 fetuses the combination of acidosis, hypoxia and propranolol resulted ... rapid deterioration of fetal circulation and fetal death 3 to 6 min after the injection ... 0.2 to 0.25 mg propranolol i v. Fig. 5 illustrates the rapid decay of MAP, FHR, maxim... dP/dt and "CO". There is also a decrease in the amplitude of the maximally high peaked T waves during hypoxia after propranolol injection, i.e. grade VI in the ECG system.

Discussion

...sults demonstrate that small doses of isoprenaline given during normoxia can elicit the sequence of events in the FECG previously recorded during hypoxia. This is in ...dance with the view that glycogenolysis induced by beta-adrenoceptor stimulation *via* ...naline will produce the changes of the FECG. Most tissues respond to oxygen lack ...reasing their rate of glycolysis, in parallel with the metabolic events elicited by beta-...tor stimulation (Mayer *et al.* 1967). In asphyxiated animals adrenaline and noradrena... may be released from the adrenal medulla leading to an increased rate of glycogen ...down due to phosphorylase activation. This mechanism has been shown to operate ...al lambs (Comline and Silver 1965). Jones and Robinson (1975) measured increased ...a catecholamine concentrations during hypoxia (fetal PaO 18 mmHg) in the chroni...y catheterized fetal lamb. They could demonstrate an increase in plasma adrenaline ...sponding to that produced by the infusion of 0.4 $\mu g\ kg^{-1}\ min^{-1}$ adrenaline. Thus, it ...ars that the dose of isoprenaline (0.02 to 0.4 $\mu g\ kg^{-1}\ min^{-1}$) which we found to elicit ...tions in the FECG pattern, is similar to the amounts of catecholamines secreted ...g hypoxia. Furthermore, an increase in the dose of isoprenaline resulted in an increase ...e duration and amplitude of the T-wave changes in parallel with the pattern of changes ...rded with increasing degree of fetal hypoxic stress. Alterations in the FECG with an ...ase in P wave and decrease in T wave amplitude together with a shortened P–R interval, ... been recorded during catecholamine infusion into fetal rhesus monkey by Adamsons ... (1971) using significantly higher dosage. Stern *et al.* also obtained higher T-waves after administration of adrenaline (100 μg i.m.) to newborn babies (1960) and to human ...ns (1961).

...oprenaline gave a positive chronotropic and inotropic effect, the later nicely demon...ted by an increase in myocardial contractility as measured by an increase in max. dP/dt. ...re was a decrease in pH and an accumulation of blood lactate during isoprenaline ...sions but no change in PaO_2. These findings are compatible with an augmented glyco...lysis. Infusion of isoprenaline had an additive effect to hypoxia and acidosis with ...ter and more pronounced FECG changes and seemed to give a more rapid deterioration ...he fetal circulation.

...he positive chronotropic and inotropic effects of isoprenaline, as well as the negative ...notropic effect of propranolol, have previously been studied in lamb fetuses (Joelsson ...al. 1972, van Petten and Willes 1970). In our study even small doses of propranolol ...1 to 0.19 mg/kg) completely abolished the FECG changes, produced either by ...prenaline or mild hypoxia. A different pattern was seen during more severe hypoxia with ...s of failing cardiovascular function. In this situation beta-blockade had no influence ... the ECG changes, consistent with the fact that asphyxia *per se* is a stimulator of phos...orylase activity in the myocardium (Mayer *et al.* 1967). The fetus was extremely suscep...le to the combination of propranolol and hypoxia. This is likely to be the result of ...propranolol blocking the most important compensatory mechanisms on the circulatory ...(...nning *et al.* 1969) as well as the metabolic side.

The question now to be asked is whether the predominant change in the FECG, th and peaked T wave, is induced by the augmentation of myocardial glycogenolysis or direct effect of the high catecholamine output on the function of the myocardial membrane. There are a number of findings in support of the former hypothesis: *A* Even during the infusion of very high doses of isoprenaline during normoxia no T wave changes were recorded. *B* Hypoxia had a marked additive effect on the of the high T waves indicating a mechanism other than beta-adrenoceptor stimulation example a direct stimulation of phosphorylase activity in the myocardium. C propranolol rapidly abolished the effect of isoprenaline, as well as the ECG ch induced by mild hypoxia, no such response was seen on the ECG during marked hy further indicating another mechanism than beta-stimulation behind the ECG chang During isoprenaline infusion there was an increase in lactate concentration which of cardiac origin (Dawes *et al* 1959)

The conclusion to be made is that mild hypoxia initiates the ECG changes activation of beta-adrenoceptors while severe hypoxia induces the ECG changes the direct effect

Thus, progressive changes in the S–T segment of the FECG during hypoxia reflect myocardial glycolysis induced by an imbalance between energy-yielding and consuming processes.

This study was supported by grants from Göteborgs Läkaresällskap, the Faculty of Medicine, G the Swedish Medical Research Council (2 591) and Prenatalforskningsfonden.

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From the Department of Environmental Medicine, Karolinska Institutet and the Department of Cardiology Södersjukhuset, Stockholm, Sweden

Respiratory and circulatory responses to sustained positive-pressure breathing and exercise in man

By

H. BJURSTEDT G. ROSENHAMER, B. LINDBORG and C. M. HESSER

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Abstract

BJURSTEDT H., G. ROSENHAMER, B. LINDBORG and C. M. HESSER. *Respiratory and circulatory responses to sustained positive-pressure breathing and exercise in man.* Acta p[illegible] scand. 1979 *105* 204-214

To investigat the effects of sustained positive-pressure breathing (PPB) on the adaptation of re[illegible] and circulatory functions to exercise 8 healthy volunteers were exposed to PPB of air at 15 and 3[illegible] in the supine position at rest and while performing leg exercise at 50 of individual maximal capacity PPB was both subjectiv ly and objectively better tolerated when combined with exercise [illegible] was at rest. PPB at 30 cm H_2O resulted in marked hyperventilation with alkalosis in the resting [illegible] but did not significantly affect respiratory minute volume, blood gases or acid-base balance during [illegible] Cardiac output and left ventricular wo k were reduced by bout one fifth and one third, respective[illegible] at rest and during exercise In contrast to the case at normal airway pressure, exercise-induced [illegible] in cardiac o tput was accompanied by n increment in stroke volume duri g PPB. Although arterial pressure (relative to tmospheric) was elevated by PPB at rest and during exercise the driving pressure [illegible] systemic circuits (arterial minus central venous pressure) was reduced in both conditions. It is [illegible] that dynamic exercise counteracts deleterious effects of PPB by normalizing respiratory function [illegible] improving cardiac f lling by activation of the leg muscle and the bdominal pumps.

Key ords. Airway pressure, exercise cardiac output, baroreflexes, arterial pressure regulation

Numerous observations are available in the literature concerning the effects of cont[illegible] positive pressure in the airways on circulatory and respiratory functions in the [illegible] condition Most of these refer to the anesthetized animal (for review see Lenfant and [illegible] 1960) Physiologic consequences in resting, conscious man which in important differ qualitatively from those in the anesthetized state, have been reviewed by Lu[illegible] Continuous positive-pressure breathing (PPB) depresses cardiac output due to obst[illegible] to venous inflow into the abdomen and chest, blood thus being displaced peripheral[illegible] distensible veins outside the trunk.

PPB is a powerful tool for the study of compensatory mechanisms that act to [illegible] homeostasis against loss of effective circulating blood volume. We are not aware [illegible]

tion is available on the combined effects of PPB and dynamic exercise. In this situa- ardiac function is taxed by increased demands on the circulation and at the same time ·inically interfered with by the increased pressure in the airways. The present experi- were designed to study the influence of PPB on the adaptation of pulmonary and vascular functions to moderate, dynamic leg exercise in supine man.

Methods

and experimental setup. 8 healthy male volunteers, aged 23–37 years, participated as test subjects formed consent was obtained. Mean body weight was 78.0 (range 65–87) kg, and mean $\dot{V}_{O_2}$ max was ange 30–75) ml kg^{-1} min^{-1} as determined from nomogram of Åstrand (1960). In all experiments, ways of the supine subjects were connected via mouth-piece, respiratory valve and large-caliber tory and expiratory tubes to ventilated pressure chamber 33 m^3 of volume. The inside pressure be controlled to give the desired mean airway pressure (0, +15 and +30 cm H_2O) within ±1 cm ventilation, tidal volume, and respiratory rate were recorded continuously on-line on breath-by- basis (Åstrom and Wigertz 1966), using signals from Fleisch flow-meter interposed on the inspira- de of the respiratory valve. Pressure in the mouth-piece was monitored and recorded continuously tal resistive pressures offered by the inspiratory and expiratory conduits amounted to 1.0 and 0.6 O at flow rate of 1.0 l s^{-1} and to 1.7 and 1.3 cm H_2O at flow rate of 2.0 l s^{-1} respectively ate was recorded from chest electrodes by means of linear, beat-to-beat heart-rate meter (Lind- ström and Wigertz 1969). Intraarterial pressure was recorded from Teflon catheter in radial mean arterial pressure was derived electronically. The central venous pressure as recorded via catheter introduced via cubital vein with its tip in or near the right atrium. Cardiac output as ured by the dye-dilution method (indocyanine green, Cardio-Green®) as described elsewhere (Bjur- Leontsteiner and Tyrfelt 1977), using the central venous catheter for dye injections. Central blood was calculated from the dye-dilution curves in accordance with the Stewart Hamilton formula. l blood gases and acid–base balance were measured with calibrated electrodes at 37°C (Instrumenta- boratory or Radiometer) in spot-samples draw from the radial-artery catheter

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To investigate the effects of sustained positive-pressure breathing (PPB) on the adaptation of respiratory and circulatory functions to exercise, 8 healthy volunteers were exposed to PPB of air at 15 and 30 cm H_2O in the supine position at rest and while performing leg exercise at 50 % of individual maximal capacity. PPB was both subjectively and objectively better tolerated when combined with exercise than at rest. PPB at 30 cm H_2O resulted in marked hyperventilation with alkalosis in the resting condition, but did not significantly affect respiratory minute volume, blood gases or acid–base balance during exercise. Cardiac output and left ventricular work were reduced by about one fifth and one third, respectively, at rest and during exercise. In contrast to the case at normal airway pressure, exercise-induced rise in cardiac output was accompanied by an increment in stroke volume during PPB. Although mean arterial pressure (relative to atmospheric) was elevated by PPB at rest and during exercise, the driving pressure of the systemic circuits (arterial minus central venous pressure) was reduced in both conditions. It is concluded that dynamic exercise counteracts deleterious effects of PPB by normalizing respiratory functions and improving cardiac filling by activation of the leg muscle and the abdominal pumps.

Key words: Airway pressure, exercise, cardiac output, baroreflexes, arterial pressure regulation

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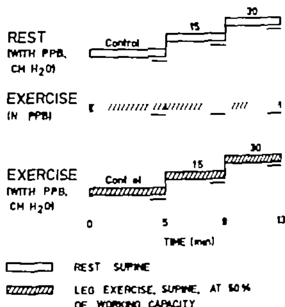


Fig. 1 Protocol showing the conditions of the experiment. PPB = continuous positive-pressure breathing. Middle portion of diagram signifies exercise at normal airway pressure. All data given in this refer to periods indicated by the short horizontal lines appearing under each condition.

Results

The subjects were allowed to breathe spontaneously throughout the protocol with no instructions given regarding respiratory rate or depth of breath. Since all subjects had previously been familiarized with PPB at rest and during exercise, it can be assumed that factors such as apprehension or anxiety were not involved in the responses observed. The absence of such emotional factors is borne out by the resting heart rates (Table II). Subjects uniformly reported moderate respiratory discomfort at the end of PPB at 30 cm H_2O in the resting condition, whereas PPB during exercise was experienced as less unpleasant than at rest.

Respiratory and gas exchange variables Table I shows the changes observed in respiratory and blood gas variables, at rest and during exercise, as the airway pressure was raised to 15 and 30 cm H O During PPB at 30 cm H_2O in the resting condition, the respiratory minute volume increased in all subjects, average increase amounting to 10 6 liters per minute or 147%, and being almost entirely due to enlargement of the tidal volume. Fig. 2 shows that the PPB-induced hyperventilation and consequent arterial hypocapnia that occurred at rest were not present in the exercise condition exercise-induced changes in respiratory minute volume, blood gases, and acid–base balance were not significantly affected by PPB (*cf* Table I)

Cardiovascular variables Table II gives the data obtained for cardiovascular variables with a change in the airway pressure from normal to 15 and 30 cm H_2O at rest and during exercise. PPB at 30 cm H_2O caused a curtailment of cardiac output averaging 22 and [illegible] at rest and during exercise, respectively It is evident from Table II and Fig. 3 that these changes were due to reductions in stroke volume, which were not fully compensated by the concurrent increases in heart rate. The calculated central blood volume fell

I. Changes in respiratory variables, blood gases and acid–base balance produced by positive airway pressure (15 and 30 cm H_2O) in the resting condition and during leg exercise at 50 per cent of aerobic working capacity

le	Rest			Exercise			Diff. Exercise—Rest at normal airway pressure
	Control	+15 cm H_2O^a	+30 cm H_2O^a	Control	+15 cm H_2O^a	+30 cm H_2O^a	
mn. volume, BTPS	7.58 ±0.49	3.78 ±2.00	10.64 ±2.29*	44.09 ±4.40	1.29 ±1.08	1.28 ±1.45	36.51 ±3.91
olume, l	0.507 ±0.033	0.291 ±0.135	0.691 ±0.186	1.925 ±0.130	0.345 ±0.217	0.420 ±0.201	1.418 ±0.122*
rate, min⁻¹	15.2 ±0.9	0.5 ±1.1	0.6 ±0.9	22.6 ±0.9	−2.2 ±1.7	−3.1 ±2.5	7.4 ±1.0***
O_2	91.4 ±3.9	17.6 ±4.9*	24.3 ±6.4	93.2 ±2.8	2.1 ±2.5	0.4 ±4.1	1.8 ±1.7
CO_2	39.8 ±1.3	−5.7 ±1.5**	−9.6 ±2.2**	40.3 ±1.6	−1.2 ±0.6	−0.2 ±1.0	0.5 ±0.6
H,	7.390 ±0.008	0.049 ±0.014**	0.089 ±0.024	7.353 ±0.010	0.010 ±0.005	−0.010 ±0.006	−0.037 ±0.004***
bicarb l	24.5 ±0.3	0 ±0.3	0.1 ±0.4	22.6 ±0.5	−0.1 ±0.2	−0.6 ±0.4	−1.9 ±0.2***

are means ± S.E. (n 8) ** and denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively ence from control.

II. Changes in circulatory variables produced by positive airway pressure (15 and 30 cm H_2O) in the resting condition and during leg exercise at 50 per cent of aerobic working capacity

ble	Rest			Exercise			Diff. Exercise—Rest at normal airway pressure
	Control	15 cm H_2O^a	+30 cm H_2O^a	Control	+15 cm H_2O^a	30 cm H_2O^a	
rate, min⁻¹	63.2 ±2.4	4.6 ±2.4	16.1 ±4.6	122.9 ±2.8	5.0 ±1.8	8.3 ±2.4	59.7 ±3.6
ac output, ⁻¹	8.76 ±0.40	−1.23 ±0.44	1.93 ±0.79*	18.12 ±0.82	−1.46 ±0.67	3.86 ±0.91**	9.36 ±1.00***
e volume,	140.5 ±6.1	27.5 ±5.5**	54.5 ±11.2*	148.6 ±5.7	−16.1 ±6.8	−34.9 ±9.3	8.1 ±4.6
mean press., Hg	91.5 ±2.9	3.6 ±1.4	7.8 ±2.1	122.9 ±4.5	1.6 ±2.0	5.3 ±2.1	24.4 ±3.5***
ven. press., H_2O 6	5.8 ±0.3	6.3 ±0.5***	15.3 ±1.0***	5.7 ±0.6	10.5 ±0.9*	21.5 ±1.3	−0.1 ±0.5
r blood volume,	1 130 ±99	180 ±67*	−291 ±108	1 097 ±113	−91 ±71	−243 ±95*	347 ±80**
ventr. ork, 6	1.02 ±0.16	0.41 ±0.10*	0.69 ±0.13	4.93 ±0.31	−0.75 ±0.26	1.62 ±0.18***	3.11 ±0.33*
periph. resist., 6	0.654 ±0.027	0.137 ±0.025**	0.373 ±0.021***	0.383 ±0.022	0.015 ±0.013	0.073 ±0.025	−0.269 ±0.036

are means ± S.E. (n = 8) and ** denote p 0.05, p 0.01, and p 0.001, respectively ence from control.

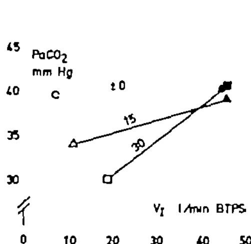


Fig.

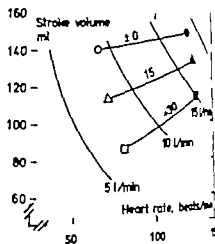


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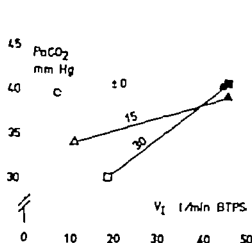


Fig. 2

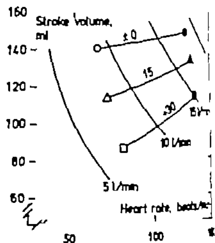


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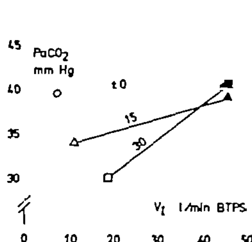


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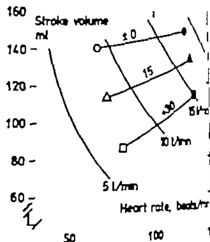


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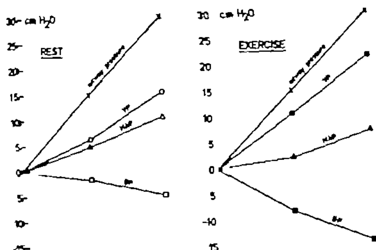
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In the calculation of the central blood volume (CBV), values for mean transit employed that referred to the transport of dye from the right atrium to the the pinna of the ear The data for calculated CBV given in Table II therefore principle, from the same uncertainty as is always the case when a peripheral employed As shown by Gleason *et al* (1959) and Marshall and Shepherd (1961) CBV will be overestimated when flow in arteries leading to the sampling site relative to that through other systemic circuits. Since in exercise the flow of blood supplying the head is greatly diminished in relation to that distributed to the muscles, the observed increase in CBV in response to exercise at normal airway which averaged 347 ml may therefore in part or even wholly have been due to of the so-called systemic component

When the airway pressure is increased in the resting condition a displacement of volume out of the thorax into distensible peripheral veins can be expected and estimated by Fenn *et al* (1947) on the basis of a caudad shift of weight in humans on a tilt table There are no data available on PPB-induced changes in CBV from ments where the dye-dilution technique has been employed with the sampling near the aortic valves. In the present experiments, PPB at 30 cm H_2O reduced CBV by an average of 291 ml at rest the corresponding reduction during exercise ml (p in both cases 0 05) No measurements are available as to the effects of PPB relative distribution of blood flow to the head and other systemic vascular beds reason the calculated reductions in CBV due to PPB should be interpreted with However since the sampling site was relatively near the root of the aorta, and it assumed that PPB did not induce any large shifts in the relative distribution of in systemic circuits, the observed reductions in calculated CBV would seem to be representative for the true changes that occurred in the blood volume contained in the and lungs

Responses of vascular pressures to PPB In the abdominal and cerebrospinal cavities the driving



Changes in measured mean arterial (MAP − 8) and central venous pressure (CVP − 6) and in ...ed driving pressure in the systemic circuits of the body DP (= MAP − CVP) as produced by in... in airway pressure. MAP represents transmural arterial pressure at the carotid baroreceptors, DP ...responding pressure at the aortic baroreceptors.

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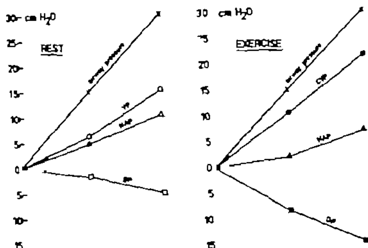
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[Ch]anges in measured mean arterial (MAP ∼ 0) and central venous pressure (CVP ∼ 0) and in [the] driving pressure in the systemic circuits of the body DP (= MAP − CVP) as produced by in[creased] airway pressure. MAP represents transmural arterial pressure at the carotid baroreceptors, DP [corres]ponding pressure at the aortic baroreceptors.

[b]etween measured mean arterial and central venous pressures (MAP and CVP respec[tively]). In the circulation outside these cavities venous blood from the periphery encounters [ele]vated pressure during PPB leading to increased transmural venous pressures with [conse]quent peripheral pooling of blood. Also in this part of the circulation DP is the dif[ferenc]e between MAP and CVP assuming that the pressures in the veins where they enter [the tr]unk approximate CVP. Hence, Fig. 4 represents the average changes observed in [MAP] and CVP and in those of DP in all systemic circuits as the airway pressure was increased [from n]ormal to 15 and 30 cm H_2O. It can be seen that DP was reduced by PPB, the respective [decre]ases at 30 cm H_2O averaging 4 mmHg at rest, and 11 mmHg during exercise (p 0.01). [Du]ring PPB the heart operates from a higher than normal pressure base. This means that [the l]eft ventricle can cut down its work, if it is to deliver the normal pressure to regions [outsi]de the trunk. In fact, PPB at 30 cm H_2O reduced left ventricular work by 37 and 33% [at re]st and during exercise, respectively, even though the mean arterial pressure (relative [to th]e atmosphere) was increased in both conditions (cf. Table II). In Fig. 4 the difference [betw]een mean arterial pressure (MAP) and the driving pressure (DP) signifies the reduction [in th]e share in MAP contributed by the contractions of the heart.

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peripheral resistance. Fig. 4 shows, on the other hand that the aortic baroreceptors as been involved. For DP represents not only the driving pressure it also approximates transmural aortic pressure assuming that CVP follows the intrathoracic pressure (cf *et al* 1946) Thus, a fall of DP induced by PPB would lead to a reduced impulse traffic the aortic baroreceptors and a consequent increase in peripheral resistance. PPB also central blood volume (*cf* Table II) and presumably also the effective filling pressure the heart due to the displacement of blood volume into peripheral veins. The vasoconstrictor effect known to result in man from unloading of intrathoracic low-pressure baroreceptors (Zoller *et al* 1972, Johnson *et al* 1974) can therefore be assumed to have acted in concert with a similar effect from aortic reflex influence. Also, the pulse pressure was presumably diminished at both the carotid and aortic baroreceptors secondary to the curtailed cardiac output which would have added to this effect (*cf* Ead, Green and Neil 1952 recent review see Kirchheim 1976) The fact that DP fell somewhat means that the otherwise marked increase in total peripheral resistance was not enough to compensate for the primary circulatory effect of PPB, that of decreased cardiac output However with the support the increased intrathoracic pressure transmitted along the arteries leading out of the thorax the vasoconstrictor defense was effective in actually increasing arterial pressure relative to the atmospheric, as illustrated by the response of MAP in Fig. 4 These responses were occurred in the remarkable situation of opposite transmural pressure changes in the carotid sinus and the aortic arch (*cf* MAP and DP in Fig. 4) a salient feature in PPB.

It is of interest to note, in this connection, the behavior of the arterial pressure in anesthetized animals during PPB In such animals, even a small increase in airway pressure by 10–15 cm H_2O leads to a fall in arterial pressure instead of the increase commonly observed in conscious man This discrepancy has been resolved since the important role of the abdominal pressure in the arterial pressure response has been recognized (Bjurstedt 1953 Bjurstedt, Wood and Åström 1953) The reduced muscle tone in the abdominal wall due to general anesthesia, or brought about by voluntary relaxation in conscious man favors venous pooling in the abdomen in addition to that in the periphery and so leads to failure of arterial pressure homeostasis.

The PPB-induced rise in total peripheral resistance at 30 cm H_2O decreased from an average of 42% at rest to 22% during exercise (p for the difference <0.01), the relative reductions in cardiac output being similar in the two conditions (22 and 21%, respectively) Exercise may therefore be regarded as having caused a weakening of the reflex vasoconstrictor influence arising from PPB-induced unloading of aortic and intrathoracic low-pressure baroreceptors. With regard to the arterial baroreceptor reflexes, certain investigators have concluded that these reflexes are reset or inhibited during exercise (for recent review, McRitchie *et al* 1976). On the other hand, a reduction of PPB-induced vasoconstriction occurring in exercise may only indicate that the vasoconstrictor outflow to resting muscles normally resulting from dynamic exercise (*cf* Blair Glover and Roddie 1961 Bevegård and Shepherd 1966) is but little influenced by the additional stimulus of PPB.

Cerebral circulation As first shown by Hamilton, Woodbury and Harper (1944), increased intrathoracic pressure as induced in a variety of respiratory maneuvers is freely transmitted to the cerebrospinal fluid due to the movements of loose tissue and fluid through the

ral foramina and into the cerebrospinal canal (for review see Scharpey-Schafer 1965). ore, PPB-induced elevation of central venous pressure will not lead to damming up od in intracranial veins. It is conceivable that PPB at a sufficiently high level could the driving pressure in cerebral circuits to such an extent that respiratory and cardio- ar effects would result from underperfusion of medullary centers. In the present however the reductions in cerebral driving pressure, represented by the fall of DP 4 were too small to make such effects likely

conclusion, sustained PPB in the supine body position reduced the driving pressure in ae circuits both at rest and during exercise. However exercise counteracted certain rious effects of PPB on various respiratory acid–base and cardiovascular functions by alizing respiratory activity and a gmenting the stroke volume. The possibility exists dynamic leg exercise, by activating both the leg muscle and the abdominal pumps, may t exposure to sustained PPB at levels that would otherwise lead to prompt failure of atory homeostasis

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peripheral resistance. Fig. 4 shows, on the other hand that the aortic baroreceptors [illegible] been involved. For DP represents not only the driving pressure, it also approxim[illegible] transmural aortic pressure assuming that CVP follows the intrathoracic pressure [illegible] *et al* 1946) Thus, a fall of DP induced by PPB would lead to a reduced impulse traffic [illegible] the aortic baroreceptors and a consequent increase in peripheral resistance. PPB [illegible] central blood volume (*cf* Table II) and presumably also the effective filling pressure [illegible] the heart due to the displacement of blood volume into peripheral veins. The vasoconstr[illegible] effect known to result in man from unloading of intrathoracic low-pressure barore[illegible] (Zoller *et al* 1972, Johnson *et al* 1974) can therefore be assumed to have acted in co[illegible] with a similar effect from aortic reflex influence. Also the pulse pressure was pro[illegible] diminished at both the carotid and aortic baroreceptors secondary to the curtailed cardiac output which would have added to this effect (*cf* Ead, Green and Neil 1952 [illegible] recent review see Kirchheim 1976) The fact that DP fell somewhat means that the [illegible] marked increase in total peripheral resistance was not enough to compensate for the pr[illegible] circulatory effect of PPB, that of decreased cardiac output However with the supp[illegible] the increased intrathoracic pressure transmitted along the arteries leading out of the [illegible] the vasoconstrictor defense was effective in actually increasing arterial pressure re[illegible] to the atmospheric, as illustrated by the response of MAP in Fig. 4 These responses [illegible] occurred in the remarkable situation of opposite transmural pressure changes in the c[illegible] sinus and the aortic arch (*cf* MAP and DP in Fig. 4), a salient feature in PPB.

It is of interest to note, in this connection, the behavior of the arterial pressure [illegible] esthetized animals during PPB In such animals, even a small increase in airway pres[illegible] by 10–15 cm H_2O leads to a fall in arterial pressure instead of the increase com[illegible] observed in conscious man. This discrepancy has been resolved since the importance of the abdominal pressure in the arterial pressure response has been recognized (Bj[illegible] 1953 Bjurstedt Wood and Åström 1953). The reduced muscle tone in the abdominal due to general anesthesia or brought about by voluntary relaxation in conscious [illegible] favors venous pooling in the abdomen in addition to that in the periphery and so lea[illegible] failure of arterial pressure homeostasis.

The PPB-induced rise in total peripheral resistance at 30 cm H_2O decreased from [illegible] average of 42 % at rest to 22 during exercise (p for the difference 0 01), the [illegible] reductions in cardiac output being similar in the two conditions (22 and 21 %, respective[illegible] Exercise may therefore be regarded as having caused a weakening of the reflex vasocon[illegible] tor influence arising from PPB-induced unloading of aortic and intrathoracic low-pre[illegible] baroreceptors. With regard to the arterial baroreceptor reflexes, certain investigators [illegible] concluded that these reflexes are reset or inhibited during exercise (for recent review, [illegible] McRitchie *et al* 1976) On the other hand, a reduction of PPB-induced vasoconstr[illegible] occurring in exercise may only indicate that the vasoconstrictor outflow to resting [illegible] normally resulting from dynamic exercise (*cf* Blair Glover and Roddie 1961 Bevegård and Shepherd 1966) is but little influenced by the additional stimulus of PPB.

Cerebral circulation As first shown by Hamilton, Woodbury and Harper (1944), increased intrathoracic pressure as induced in a variety of respiratory maneuvers is freely transmitted to the cerebrospinal fluid due to the movements of loose tissue and fluid through the [illegible]

Acta physiol. scand. 1979 105, 215–221

From the Department of Physiology University of Goteborg, Sweden

In vitro effects of growth hormone on protein synthesis and amino acid transport in the rat diaphragm after acute hypophysectomy

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Received 5 June 1978

Abstract

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Hypophysectomy is, however accompanied by a hormonal imbalance which might use structural and metabolic aberrations in the tissues (Everitt 1976). Florini and Breuer 966) demonstrated that the capacity of thigh muscle ribosomes of chronically hypo-

physectomized rats to catalyze protein synthesis was markedly reduced. Further single dose of GH was found to restore the capacity of the ribosomes to that of n after a time lag of several hours. The yield of ribosomal RNA paralleled the These findings suggest that the long duration of the effects of GH on protein metab hypophysectomized rats might not be representative for normal rats but merely a of a functional impairment in the protein synthetic machinery due to the chronic lack

In the present study the effects of exogenous GH on protein metabolism of the dia muscle of the rat were studied at short time intervals after hypophysectomy e.g. lack of pituitary hormones would cause structural changes in the peripheral tissues

Materials and Methods

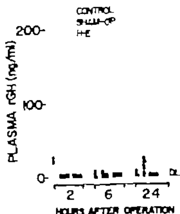
Animals. Female rats, 30 days old (80–95 g) of the Sprague-Dawley strain purchased from Anticimex Stockholm, Sweden were used in all experiments. The animals were housed in a room in which l maintained from 05 00 to 19 00 h and temperature (25 C) and humidity (50–60) were kept The rats were fed standard commercial diet (Typo R3 Anticimex, Stockholm), and tap water The rats were hypophysectomized under ether anesthesia by the standard parapharyngeal approach 1930) that has been used for ma y y ars in this laboratory (*e.g.* Hjalmarson 1968). A corresponding of rats were sham-operated—that is all steps in the surgical procedure e cept removing the pituitary were performed. After the operation the rats were kept in the animal room and received food and in addition 50 glucose sol tion *ad libitum*. One group of the rat were untreated and used as , 6 o 4 h fter the operation the rats were k lled for *in vitro* experiments.

Radioact materials Radioacti substances, obtained from New England Nuclear Corp. Mass., were used at th f llowing activities nd molarities. α-amino-isobutyric acid-1 ^{3}H (U) μCi/ml, 0.2 mM L-phenylalanine- ^{14}C (U), 0.05 μCi/ml, 0.08 mM

Hormone Bovine growth hormone [NIH bovine GH 18 (bGH)] with a potency of 0 8 IU/mg was by the National Institute f Arthritis, Metabolism and Digestive Diseases (NIAMDD).

Incubation procedure Rats were rapidly decapitated and blood was collected i chilled beakers for subsequent glucose and GH determination. I tact hemidiaphragms were prepared nd Knobil 1959) nd washed for 10 mi at 37°C in 5 ml Krebs bicarbonate buffer pH 7.4 (KRB) librated with 95 O_2 and 5 CO After the washi g period, nd vidual hemidiaphragms were to other flasks containi g 10 ml of fresh KRB with ll natural amino acids at normal plasma levels *et al* 1971) nd the labelled amino acids. The medium for one numbe of each pair of muscles also bGH (5 μg ml). The hemidiaphragms were incubated at 37°C for 60 min and then the muscles in buffer weighed and homogenized in 1.5 ml 5 perchloric acid. The amount of protein was according to Lowry *et al.* (1951). Radioactivity measurements were performed as previously (Albertsson-W kland and Isaksso 1976). The extent of amino acid transport was calculated distributi n ratio (cpm/ml intracellular water cpm/ml incubation medium) usi g values for cell previously determined (Hjalmarson and Ahrén 1967 *a*, Albertsson-Wikland and Isaksson 1976) alanine incorporation was e pressed as dpm of ^{14}C incorporated/100 μg of muscle protein.

Plasma GH determination Blood collected from the trunk f decapitated rats was immediately and plasma was separated, stored nd frozen t − 30°C until assay Plasma GH was measured immunoassay using a double a tibody tech ique as previously described (Edén *et al* 1978). A preparation, highly purified rat GH especially prepared by D A. E. Wilhelmi with an activity of was used The same h rmone was labelled with I ^{125} according to the lact peroxidase method (Thorell Johansson 1971). Monkey anti-rGH-serum (A rGH S-3) was generously supplied by the N Precipitating antibodies against normal monkey serum were raised in rabbits according to the of V itukaitis *et al* (1971). Triplicates of plasma i 3 dilutions (5 10 and 20 μl/tube) were determination of the rGH concentration. The olume f plasma was k pt constant in all samples equivalent amounts of plasma from hypophysectomized rats. The detection limit of the assay plasma (0 05 g rGH/t be) nd the standard curve was linear between 5–200 ng/ml plasma rGH/tube).



[illegible]lasma levels of GH in normal, sham-operated (SHAM-[illegible] hypophysectomized (H-E) rats. Rats were rapidly [illegible]ed and blood collected from the trunk. Plasma GH [illegible]sured by radioimmunoassay. D.L. represents the detec[illegible] (2 S.D. abov[illegible] zero dose in the standard curve). Each [illegible]presents the plasma level of GH in individual rats. There [illegible] measurable GH-levels in hypophysectomized rats. In [illegible]erated rats GH-levels [illegible]re undetectable 2 h after sur[illegible] [illegible]ficantly lower than in normal rats 6 h after surgery [illegible] (Mann Wilcoxon [illegible] test) but not significantly different [illegible]mal levels 24 h after surgery.

[illegible]al procedures. Values are given as means ± S.E. Comparisons were made by paired t-test, or [illegible]re than 2 values [illegible]re compared, by analysis of variance followed by Student Newman-Keuls' [illegible] test between individual groups (Woolf 1968). P-values less than 0.05 were considered significant [illegible] [illegible]y. When plasma GH levels were compared Wilcoxon [illegible] non-parametric test [illegible] used (Colquhoun

Results

[illegible] levels of GH were measured in all rats. There were no detectable levels of GH 2, 6 [illegible]h after hypophysectomy. Sham-operation caused a marked reduction of plasma GH (Fig. 1). There were no detectable levels of GH 2 h after sham-operation. 6 h post[illegible] [illegible]ely 4 out of 12 animals had measurable plasma GH levels but levels were signifi[illegible] lower compared to normal controls. 24 h after sham-operation GH levels were almost [illegible]d to normal levels.

[illegible]d glucose was also measured in all rats (Table I). The table shows that there was a [illegible]ncrease in blood glucose 2 h postoperatively in sham-operated and hypophysectomized [illegible] h postoperatively there were no significant differences between the three groups. [illegible]fter hypophysectomy blood glucose levels were slightly decreased.

[illegible] effects of bGH (5 μg/ml) *in vitro* on the incorporation of phenylalanine [illegible]C into [illegible] and on the accumulation of AIB-[illegible]H in diaphragms of normal, sham-operated and [illegible]physectomized rats [illegible] h postoperatively are shown in Fig. 2. GH did not stimulate these

[illegible] Blood glucose in normal, sham-operated and hypophysectomized rats 2, 6 and 24 h postopera[illegible]tively

[illegible]		Blood glucose (mM)		
		normal rats	sham-operated rats	hypophysectomized rats
	12	5.53 ± 0.43	6.74 ± 0.24	6.50 ± 0.46*
	11	5.43 ± 0.38	6.00 ± 0.21	5.23 ± 0.38
	10	5.85 ± 0.48	5.79 ± 0.26	4.03 ± 0.41

[illegible]05 as control with analysis of variance.

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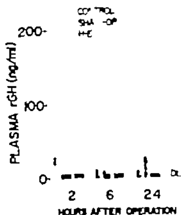
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Animal Female rats, 30 days old (80-95 g) of the Sprague-Dawley strain purchased from Anticimex Stockholm Sweden, were used in all experiments. The animals were housed in a room in which [illegible] maintained from 05 00 to 19 00 h and temperature (25°C) and humidity (50-60) were held [illegible] The rats were fed a standard commercial diet (Type R3 Anticimex Stockholm), and tap water [illegible] The rat were hypophysectomized under ether anesthesia by the standard parapharyngeal approach [illegible] 1930) that has been used f r many years in this laboratory (*e g* Hjalmarson 1968). A corresponding [illegible] f rat were sham-operated—that is all steps in the surgical procedure except removing the pituitary were perf rmed Afte the operation the rat were kept in the animal room and received food [illegible] nd in add tion 50 [illegible] glucose sol tion *ad libitum*. One group of the rats were untreated and used [illegible] [illegible], 6 o 4 h fter the operation the rats were killed fo *in vitro* experiments.

Radioactive materials Radioactive subst nces, bta ned from New England Nuclear Corp [illegible] Mass., were used at the following acti ities nd molarities. α-am no-isobutyric acid-1 [illegible] (AIB) μCi/ml 0.2 mM L phenylalanine [illegible]C (U), 0 05 μCi/ml, 0.08 mM

Hormone Bovine growth hormon [NIH-bovine GH 18 (bGH)] with a potency of 0 8 IU/mg was [illegible] by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD).

Incubation procedure Rats were rapidly decapitated and blood was collected in chilled [illegible] beakers fo subsequent glucose nd GH determination. I tact hemidiaphragms were prepared [illegible] and Knobil 1959) and washed f r 10 min at 37 C in 25 ml Krebs bicarbonate buffer pH 7 4 (KRB) librated with 95% O and 5 CO After the wash ng period, ndividual hemidiaphragms [illegible] to othe flasks cont ining 10 ml f fresh KRB with all natural amino acids t normal plasma levels [illegible] *et al* 1971) nd the labelled amino acids. The medium fo one number of each pair of muscles also [illegible] bGH (5 μg/ml). The h midiaphragms were incubated t 37°C f r 60 mi and then the muscles [illegible] buffer weighed and homogenized in 1 5 ml 5 perchlor c cid The mount of protein was determined according to Lowry *et al.* (1951). Radioactivity measurement were performed as previously [illegible] (Albertsson-Wikland and Isaksson 1976) The extent of amino acid transport was calculated as the distribution ratio (cpm/ml i tracellular water cpm/ml incubatio medium) using values for cellular [illegible] previously determined (Hjalmarson and Ahrén 1967 Albertsson W kland nd Isaksson 1976) [illegible] ala ine incorporation was pressed as dpm of C incorporated/100 μg of muscle prot in.

Plasma GH determination Blood coll cted from the trunk of decapitated rats wa immediately [illegible] and plasma was separated stored and frozen t 30°C until assay Plasma GH wa measured [illegible] immunoassay using a doubl tibody technique s previo sly described (Edén *et al.* 1978). A [illegible] preparation, highly purified rat GH especially prepared by Dr A. E. Wilhelmi with an activity of [illegible] was used. The same hormone was labelled with I [illegible] according to the lactopero idase method (Thorell and Johansson 1971). Monkey nti-rGH-serum (A-rGH S-3) was generously supplied by the NIAMDD. Precipitating antibodies against normal monkey serum w re aised i rabbits according to the [illegible] of Vait kaitis *et al* (1971) Triplicates f plasma in 3 dilutions (5 10 nd 20 μl/t be) were used [illegible] determination of the rGH concentration. The volume of plasma was k pt constant in all samples by [illegible] equivalent amounts of plasma from hypophysectomized rats. The detection limit of the assay was [illegible] plasma (0.05 ng rGH/t be) a d the standard curve was line r between 5-200 ng/ml plasma (0 [illegible] rGH/tube).



Effect of GH *in vitro* on phenylalanine-^{14}C incorporation (left) and AIB-^{3}H accumulation (right) in [illegible] of normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 24 h after surgery [illegible] diaphragms were incubated for 60 min with and without bGH (5 µg/ml). The incorporation of phenyl-[illegible]-^{14}C into muscle protein and the distribution ratio of AIB-^{3}H were determined as described in [illegible]ds. Each bar represents the mean of 10–12 observations. Vertical lines represent S.E.

0.01 cr normal control with analysis of variance
0.001 cr normal control with analysis of variance
0.001 cr corresponding control with paired t-test

[illegible]tion in diaphragms of both sham-operated and hypophysectomized rats, but had no [illegible] in the control group of normal rats. In the hypophysectomized group, GH *in vitro* [illegible]st restored phenylalanine-^{14}C incorporation to normal levels while the effect was less [pr]onounced but significant in the sham-operated group. The distribution of AIB-^{3}H was [not s]ignificantly altered in the sham-operated group while the distribution was markedly [incr]eased in diaphragms of hypophysectomized rats. GH *in vitro* did not significantly [stim]ulate amino acid transport in normal or sham-operated rats whereas the distribution of AIB-^{3}H was further elevated in diaphragms of hypophysectomized rats.

[24] h postoperatively (Fig. 4) the incorporation of phenylalanine-^{14}C and the accumulation [of A]IB-^{3}H was markedly decreased in diaphragms of hypophysectomized rats. Exposure [to G]H *in vitro* resulted in a marked stimulation of both of these parameters. Thus, the in[corp]oration rate of phenylalanine-^{14}C and accumulation rate of AIB-^{3}H were close to that in [dia]phragms of normal rats. In the sham-operated rats the distribution ratio of AIB-^{3}H [was s]lightly depressed but the incorporation of phenylalanine-^{14}C was unchanged compared [to d]iaphragms of normal rats. GH did not significantly affect any of these two parameters [in t]he sham-operated group.

Discussion

[The i]mmediate metabolic changes following hypophysectomy have received little attention. [Goodn]er and Russell (1968) found an increase in nitrogen excretion and a decrease in the [car]diac glycogen content 24 h after hypophysectomy in the rat. They further found that GH [the]rapy restored these parameters to control values suggesting that the observed phenomenon [is] partially due to lack of GH.

The present study clearly shows that the processes regulating the rate of amino acid [tra]nsport and protein synthesis become responsive to exogenous GH a short time after [hypophys]ectomy. In the present experiments significant effects of GH on these two para-

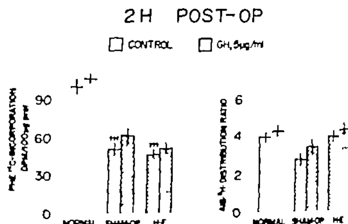


Fig. 2. Effect of GH *in vitro* on phenylalanine-14C incorporation (left) and AIB-3H accumulation in diaphragms of normal sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 2 h after surgery. Hemidiaphragms were incubated for 60 min with and without bGH (5 µg/ml). The incorporation of phenylalanine-14C into muscle protein and the distribution ratio of AIB-3H were determined as described in Methods. Each bar represents the mean of 10–12 observations. Vertical lines represent S.E.

† $p < 0.05$ *vs.* normal control with analysis of variance
††† $p < 0.001$ *vs.* normal control with analysis of variance

two parameters in any of the groups studied However there was a marked decrease in control levels of phenylalanine-14C incorporation in diaphragms of sham-operated and hypophysectomized rats compared to normal rats. The accumulation of AIB-3H was also decreased in the control diaphragms of sham-operated rats but not in diaphragms of hypophysectomized rats

6 h after surgery the incorporation of phenylalanine-14C was still reduced in both operated groups (Fig 3). However GH *in vitro* stimulated the incorporation of the amino acid

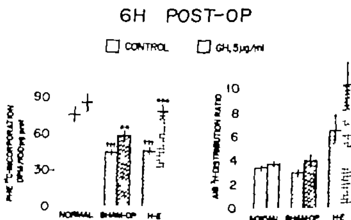
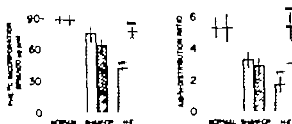


Fig. 3 Effect of GH *in vitro* on phenylalanine-14C incorporation (left) and AIB-3H accumulation in diaphragms of normal, sham-operated (SHAM-OP) and hypophysectomized (H E) rats 6 h after surgery. Hemidiaphragms were incubated for 60 min with and without bGH (5 µg/ml). The incorporation of phenylalanine-14C into muscle protein and the distribution ratio of AIB-3H were determined as described in Methods. Each bar represents the mean of 10–12 observations. Vertical lines represent S.E.

† $p < 0.05$ *vs.* normal control with analysis of variance
††† $p < 0.001$ *vs.* normal control with analysis of variance
$p < 0.05$ *vs.* corresponding control with paired t test
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$p < 0.001$ *vs.* corresponding control with paired t-test



Effect of GH in vitro on phenylalanine-^{14}C incorporation (left) and AIB-^{3}H accumulation (right) in [diaphra]gms of normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 24 h after surgery. [Di]aphragms were incubated for 60 min with and without bGH (5 μg/ml). The incorporation of phenyl[alanine]-^{14}C into muscle protein and the distribution ratio of AIB-^{3}H were determined as described in [Metho]ds. Each bar represents the mean of 10–12 observations. Vertical lines represent S.E.

[p < 0.]01 vs normal control with analysis of variance
[p < 0.]001 vs normal control with analysis of variance
[p < 0.]001 vs corresponding control with paired t-test

[...]ns in diaphragms of both sham-operated and hypophysectomized rats, but had no [effect] in the control group of normal rats. In the hypophysectomized group, GH *in vitro* [almos]t restored phenylalanine-^{14}C incorporation to normal levels while the effect was less [pron]ounced but significant in the sham-operated group. The distribution of AIB-^{3}H was [not si]gnificantly altered in the sham-operated group, while the distribution was markedly [decre]ased in diaphragms of hypophysectomized rats. GH *in vitro* did not significantly [stimu]late amino acid transport in normal or sham-operated rats whereas the distribution [ratio] of AIB-^{3}H was further elevated in diaphragms of hypophysectomized rats.

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Discussion

[The i]mmediate metabolic changes following hypophysectomy have received little attention. [Mc]Kee and Russell (1968) found an increase in nitrogen excretion and a decrease in the [...]line glycogen content 24 h after hypophysectomy in the rat. They further found that GH [ther]apy restored these parameters to control values suggesting that the observed phenomenon [was] partially due to lack of GH.

[T]he present study clearly shows that the processes regulating the rate of amino acid [tran]sport and protein synthesis become responsive to exogenous GH a short time after [hypo]physectomy. In the present experiments significant effects of GH on these two para-

meters were seen at 6 and 24 h after hypophysectomy These results suggest that tory effects of endogenous GH on protein synthesis and amino acid transport in ... rat are of short duration in contrast to the long lasting effects of GH on the previously reported in tissues of hypophysectomized rats (Kostyo and Nutting 197 marson and Ahrén 1967 *b*) This hypothesis is further supported by recent made in our laboratory using the diaphragm muscle of normal 18-day-old rats (Albe Wikland and Isaksson 1976, 1978) In these experiments the duration of the stimul effects of GH on protein synthesis and amino acid transport was considerably pared to that previously reported in hypophysectomized rats.

From the present experiments it is not clear to what extent the changes in protein lism observed could be ascribed to lack of GH The surgical trauma *per se* most pr caused changes in tissue metabolism, which in turn might change the tissue res to GH The possibility that a substantial amount of GH leaked out into the blood during the operation, and then influenced peripheral metabolism for a long time after the operation was ruled out, since plasma levels of GH are low during hyp tomy and remain undetectable after the operation (unpublished observation).

GH levels in plasma were low or undetectable also in sham-operated rats for at le after the surgical trauma, which is in accordance with previous observations which shown that unspecific stress inhibits the release of GH (Takahashi *et al* 1971 Du 1973/74 Terry *et al* 1976) In spite of low or undetectable levels of GH in both operated and hypophysectomized animals for at least 6 h after the operation, the distri ratio of AIB was markedly elevated in the hypophysectomized group while there change in the distribution ratio of AIB in the sham-operated group. Interestingly, observations have been made in the small intestine of the rat by Levitan *et al.* (197 reported an initial increase in amino acid transport after hypophysectomy followe marked decrease. The cause of the altered amino acid uptake after hypophysecto pared to that after sham operation is not clear but this phenomenon indicates th factors besides low GH levels in plasma are involved in the metabolic changes rep after hypophysectomy

Recent studied by Martin and co-workers (Tannenbaum and Martin 1976) have that GH is secreted in a pulsatile pattern in the rat The short duration of the stim effect of GH on protein synthesis in young normal animals opens the possibility that of GH intermittently stimulate protein synthesis. Such a temporal relationship be peaks in plasma concentration of GH and protein synthesis in skeletal muscle has been proposed (Isaksson *et al* 1978) Further studies are, however required to subst this hypothesis.

The authors express their appreciation t D Kurt Ahrén for helpf l discussions and critical rea the manuscript The uthors re indepted t the NIAMDD for th bovine growth hormone prep and the NIAMDD Rat Pituitary Hormone Distributio Program, and A. F Parlow for kindly su antiserum to rat GH (NIAMDD-Anti-Rat GH Serum-3). H ghly purified rat GH (R 1051 A) was supplied by Dr A. E. Wilhelmi This research was supported by grants from the Swedish Medical Re Council (B78-14X-04250), M gnus Bergvall Foundation, Stockholm and the Faculty of Me University of Göteborg. Excellent technical assistance wa given by Miss Britt Marie Johans Miss Barbro Hemskog.

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Metabolic effects of blood flow restriction in adipose tissue

By

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Abstract

BELFRAGE E., P HJEMDAHL and B B FREDHOLM. *Metabolic effects of blood flow restriction in adipose tissue* Acta physiol scand 1979 *105* 222-227

The metabolic effect of blood flow restriction were studied in isolated blood-perfused canine adipose adipose tissue Blood flow restriction (on the average to 20 per cent of control flo) as caused by mechanical clamping of the arterial nflow or by i a injections of methoxamine or angiotensin G uptak in the dipose tissue was reduced during blood flow restriction. This was partially compensated for by a period of increased glucose ptake following restoration of flow Blood flow restriction also a increase i the venous lactate/pyruvate ratio Th basal lipolytic rate was decreased during blood restriction Lipolysis induced by brief (5 min) sympathetic nerve stimulation (4 Hz) as not inhibited blood flow restriction s the total amount of glycerol released from the tissue was unaffected. The rate was reduced duri g blood fl w restriction but glycerol trapped within the tissue was apparently reutilized by the f t cells as it was rele sed upon flow restoration FFA outflow following nerve stimulation was, however nhibited suggesting ncreased reutilization of FFA w thi the tissue This increased re-utilization may ultimat ly be caused by the observed change in red./ox.-balance and/or by the limited capacity (albumi) ava lable d ring blood fl w restriction. Three main concl sions may be drawn from the present results. Firstly plasma levels of glycerol nd FFA do not necessarily reflect adipose tissue lipolysis at given moment. Secondly the decreased ad pose tissue blood flow seems to be a major cause lowered FFA-levels d ring hemorrhage Th rdly in contrast to hemorrhage even severe reduction adipose tissue blood flow is nsufficient to cause irreversible ischemic damage

Key ords. Adipose tissue, Blood flow Esterification Fatty acids, lipolysis.

Plasma levels of glycerol and FFA (non-esterified fatty acids) are commonly used as of lipolysis and are thought to closely reflect events in the adipose tissue. It has been reported that plasma levels of glycerol are unchanged and those of FFA actually decreased during hemorrhage in dogs (Rosell *et al* 1973) and rats (Farnebo *et al* 1977). This decrease plasma FFA during hemorrhage, which is accompained by intense sympatho-adrenal activation has been attributed to the severe reduction in adipose tissue blood flow (Rosell et 1973). Kovách *et al* (1976) reduced blood flow to the unstimulated adipose tissue by 85% during 60 minutes. They found that the basal outflow of glycerol and FFA reduced during flow reduction but that no irreversible damage had been caused by experimental procedure. The ability of the adipose tissue to respon stimulus during conditions of blood flow restriction ever

d. We have therefore examined the influence of blood flow restriction, caused either oconstrictor agents or by mechanical clamping of the arterial inflow on the response standardized (Fredholm 1970) stimulation of the sympathetic nerves supplying the taneous adipose tissue of the dog.

Methods

periments were performed on female mongrel dogs weighing 10–20 kg. The dogs were anesthetized lbarbital (30 mg kg with supplements as necessary) and mechanically ventilated. Subcutaneous tissue in the inguinal region was completely isolated from the surrounding tissue (Rosell 1966), for one artery one vein and nerve containing sympathetic fibres. The vessels were cannulated with tubing and the adipose tissue was connected to the femoral circulation. Adipose tissue blood flow measured with silicon filled drop recorders on the arterial side and registered together with carotid blood pressure on a Grass 7B polygraph. Restriction of adipose tissue blood flow for 60–90 min was d by a) mechanical clamping of the arterial supply b) repetitive i.a. injections of 4–6 μg methoxamine epetitive injections of 100–300 ng angiotensin (Hypertensin CIBA) although final doses of 3–10 μg nsin was needed to maintain low flow. Nerve stimulation was performed by placing the sectioned e tissue nerve on a bipolar silver electrode giving electrical impulses at 4 Hz for 5 min with supra mal intensity (13 V) and duration (2 ms). Venous blood samples from the adipose tissue vein and l blood samples were collected into ice-cooled centrifuge tubes. Lactate and pyruvate concentrations determined in aliquots of whole blood with commercially available reagents (TCC, TCB, Boehringer ...). After centrifugation aliquots of plasma were removed for the determination of glycerol (Laurell Tibbling 1966), FFA (Laurell and Tibbling 1967) and glucose (Glox, Kabi). Uptake or release rates actate, pyruvate, glycerol, FFA and glucose were calculated by multiplying the V-A-concentration ence with adipose tissue blood or plasma flow. The net release of glycerol or FFA was obtained by acting the basal release from the total amount released during and following sympathetic nerv ulation.

Results

pose tissue blood flow was reduced (on the average by 80 per cent of the flow during rol conditions) during 90 min either by mechanical clamping of the arterial supply or

1 Net release of glycerol and FFA was calculated as the total amount released during and after sympathetic nerve stimulation. In the case of flow reduction the amounts mobilized after flow restoration have been included. In absolute terms (μmol 100 g⁻ tissue) the net release of glycerol was 18 6±3 7 (control) and 14 6±3 0 (flow reduction) and FFA was 33 8±10.1 (control) and 7 5 4 4 (flow reduction)

reduction	of control	Net release Glycerol	of control FFA
…otensin	26	104	49
…otensin	37	108	24
…hexamine	16	41	0
…hexamine	8	112	0
…hexamine	17	99	5
…mp	23	74	32
…mp	7	106	8
…mp	14	36	3
…mp	10	105	0
[illegible]	18±3	87_10	12±6

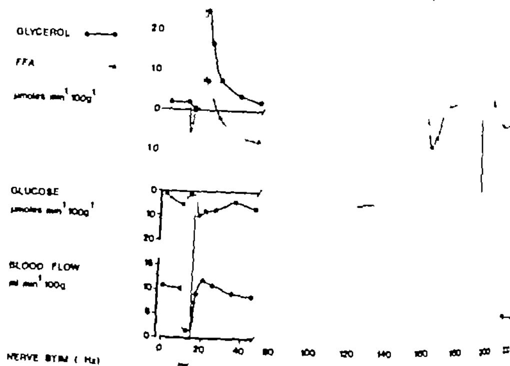


Fig. 1 Time course of changes in release/uptake rates of glycerol, FFA and glucose and blood ... one experiment. Three consecutive sympathetic nerve stimulations were performed, the second one ... during reduction of blood by mechanical clamping.

by i.a. injections of methoxamine or angiotensin (Table I) Angiotensin was used in ... 2 expts. since a sustained blood flow reduction was difficult to obtain due to tachyphyl...

In each experiment three consecutive sympathetic nerve stimulations at 4 Hz for 5 ... were performed, the first and the third serving as controls while the second stimulation ... performed during the period of flow reduction (Fig. 1) There were no differences bet... the two control periods concerning uptake/release of glucose, lactate, glycerol or FFA. Furthermore, there were no differences in net glycerol or FFA release between the ... control periods. Therefore, the events during flow reduction have been compared to ... means of the two controls in each experiment.

The basal outflow of glycerol from the adipose tissue decreased somewhat during the flow restriction (Fig. 1 Table II), even though there was a compensatory increase in ... V A glycerol concentration difference at lower blood flow rates (Fig. 2). The peak out... of glycerol following sympathetic nerve stimulation was delayed (Fig. 1) and mark... reduced by flow restriction (Fig. 1 Table II). The maximal V A concentration differ... (following stimulation) for glycerol did not increase during flow restriction, indicating ... the maximal transport capacity for glycerol is reached already after a 4 Hz nerve stimul... during control conditions. The net release of glycerol from the adipose tissue due to the brief sympathetic nerve stimulations during blood flow restriction was, however, unchan... (Table I)

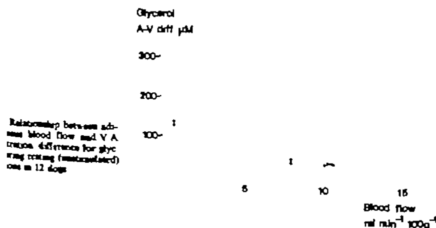
During control conditions there was a net uptake of FFA ... unstimulated adip...

Mean release rates (N = 9) of glycerol, FFA and glucose (A) at rest before sympathetic nerve stimulation (B) peak after sympathetic nerve stimulation and (C), the peak seen upon restoration of blood flow I the case of glucose uptake during period B the mean uptak during the whole 30 to 40 min period after sympathetic nerv stimulation as calculated.

	Release rate (µmol min⁻¹ 100 g⁻¹)		
	A. Mean before stim	B. Peak after stim.	C. Peak following flow restoration
al	0.18 ± 0 02	3.02 ± 0.54	—
reduction	0 09 ± 0 02	0 51 ± 0.19	0.58 ± 0.12
al	−0.29 ± 0.11	3.35 ± 0 97	—
reduction	−0 01 ± 0 04	0 41 ± 0.28	−0.37 ± 0.37
		Mean after stim.	
al	−4.2 ± 0.5	−4 1 ± 0 4	—
reduction	−1.9 ± 0.2	−2.6 ± 0 4	−8.1 ± 1.3

ig. 1 Table II), which was reduced by flow restriction. Both the peak outflow rate Table II) and the net release of FFA (Table II) after sympathetic nerve stimulation ntly diminished by flow restriction. There was no compensatory increase in FFA upon restoration of blood flow (Fig. 1 T ble II), in contrast to what was found for . Glucose uptake was reduced by approximately 50 per cent during blood flow on (Table II). After restoration of flow there was a compensatory increase in glucose which lasted 5 to 10 min (Fig 1 Table II).

ng control conditions there was a net uptake of lactate in the adipose tissue amounting ±0.35 µmol min 100 g⁻ Blood flow restriction converted this uptake into a duction rate of 0.57 ± 0.16 µmol min 100 g⁻ As a further indication of anaerobic metabolism the lactate/pyruvate concentration ratio in the venous effluent from the tissue increased from 15 ± 4 to 27 ± 8.



Relationship between adipose blood flow and V A tration difference for glyc ring resting (unstimulated) ons in 12 dogs

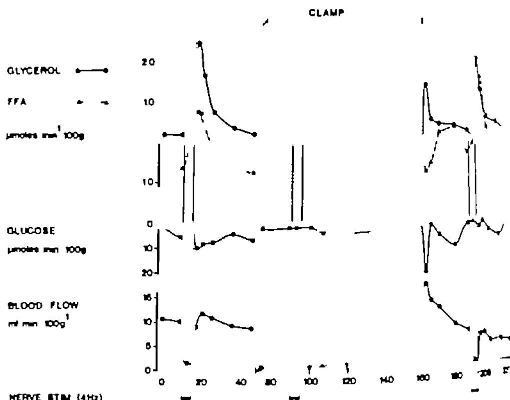


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In each experiment three consecutive sympathetic nerve stimulations at 4 Hz for ... were performed, the first and the third serving as controls while the second stimulation ... performed during the period of flow reduction (Fig. 1) There were no differences bet... the two control periods concerning uptake/release of glucose, lactate, glycerol or F... Furthermore, there were no differences in net glycerol or FFA release between the ... control periods. Therefore, the events during flow reduction have been compared to ... means of the two controls in each experiment.

The basal outflow of glycerol from the adipose tissue decreased somewhat during the ... flow restriction (Fig. 1 Table II) even though there was a compensatory increase ... V A glycerol concentration difference at lower blood flow rates (Fig. 2). The peak out... of glycerol following sympathetic nerve stimulation was delayed (Fig. 1) and mark... reduced by flow restriction (Fig. 1 Table II) The maximal V A concentration differ... (following stimulation) for glycerol did not increase during flow restriction, indicating ... the maximal transport capacity for glycerol is reached already after a 4 Hz nerve stimul... during control conditions. The net release of glycerol from the adipose tissue due to ... brief sympathetic nerve stimulations during blood flow restriction was, however unch... (Table I).

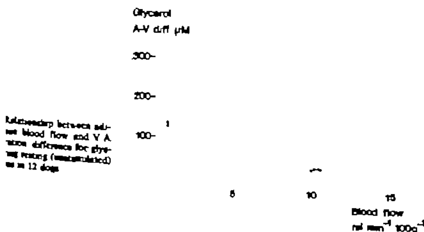
During control conditions there was a net uptake of FFA in the unstimulated ad...

Mean release rates (N 9) of glycerol, FFA and glucose (A) at rest before sympathetic nerve stimulation (B) peak after sympathetic nerve stimulation and (C), the peak seen upon restoration of blood flow In the case of glucose uptake during period B the mean uptake during the whole 30 to 40 min period after sympathetic nerv stimulation was calculated.

	Release rate (µmol min⁻¹ 100 g⁻¹)		
	A. Mean before stim.	B. Peak after stim.	C. Peak following flow restoration
l	0.18 ± 0.02	1.02 ± 0.54	—
eduction	0.09 ± 0.02	0.51 ± 0.19	0.58 ± 0.12
d	0.29 ± 0 11	3.35 ± 0.97	—
eduction	−0 01 ± 0 04	0.41 ± 0.28	−0.37 ± 0.37
		Mean after stim.	
d	−4.2 ± 0 5	−4 1 ± 0.4	—
eduction	1.9 ± 0.2	−2 6 ± 0 4	−8.1 ± 1.3

Fig 1 Table II), which was reduced by flow restriction. Both the peak outflow rate Table II) and the net release of FFA (Table II) after sympathetic nerve stimulation antly diminished by flow restriction. There was no compensatory increase in FFA upon restoration of blood flow (Fig. 1 Table II), in contrast to what was found for . Glucose uptake was reduced by approximately 50 per cent during blood flow on (Table II) After restoration of flow there was a compensatory increase in glucose which lasted 5 to 10 min (Fig. 1 Table II).

g control conditions there was a net uptake of lactate in the adipose tissue amounting ±0.35 µmol min 100 g⁻ Blood flow restriction converted this uptake into a duction rate of 0.57 ± 0.16 µmol min 100 g⁻¹ As a further indication of anaerobic metabolism the lactate/pyruvate concentration ratio in the venous effluent from the tissue increased from 15 ± 4 to 27 ± 8.



Relationship between adi- se blood flow and V A ation difference for glyc- ing resting (unstimulated) ue in 12 dogs

Discussion

The present results confirm the opinion that adipose tissue blood flow is of impor[illegible] the mobilization of lipolytic products, *i.e.* glycerol and FFA, from the tissue ([illegible] 1966 Fredholm 1970). A severe reduction of adipose tissue blood flow does not [illegible] impair the lipolytic response to sympathetic nerve stimulation, since the net glycerol was unchanged. On the other hand, the glycerol mobilization was delayed substantial part of the release occurred after the period of restricted flow. When lip[illegible] is induced in a tissue with low blood flow glycerol may therefore be trapped in the [illegible] for very long periods of time before mobilization. This implies that observed alterat[illegible] plasma glycerol not necessarily reflect the lipolytic rate in the adipose tissue at a give[illegible] Himms-Hagen (1967) has enumerated several factors that tend to make conclusions [illegible] cerning actions in adipose tissue from studies of plasma FFA and glycerol levels [illegible] The present findings seem to add yet another complicating factor to that list.

While blood flow restriction did not affect the total amount of glycerol released [illegible] period of nerve stimulation (if sufficient time for sampling was allowed for) there [illegible] reduction of the net release of FFA. This suggests that blood flow restriction has [illegible] the re-utilization of fatty acids in the tissue.

The increased utilization of FFA during blood flow restriction is best explained b[illegible] increased rate of reesterification of newly formed fatty acids. Several factors may con[illegible] to this. Firstly a low blood flow and thus a decrease in carrier capacity (*i.e.* albumi[illegible] increase the concentration of FFA within the tissue. Secondly the increased lactate/pyr[illegible] ratio indicates an elevated NADH/NAD -ratio (Huckabee 1958). This will enhan[illegible] formation of α-glycerophosphate which is the other substrate required for trigly[illegible] synthesis in the adipose tissue, as discussed previously (Fredholm 1971).

The present experiments show that the adipose tissue is not damaged by blood f[illegible] restriction during 60–90 min since stimulated glycerol and FFA-outflow as well as gl[illegible] uptake were almost identical during observation periods before and after blood flow re[illegible] tion. Similarly Kovách *et al.* (1976) found no irreversible damage to unstimulated adi[illegible] tissue when blood flow was reduced to 15% of control for 60 min by mechanical clam[illegible] Thus restriction of adipose tissue blood flow to less than 20% of control does not pro[illegible] evidence of ischemia.

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Acta physiol. scand. 1979 105. 228–238

From the Institute of Medical Physiology Dept. A. University of Copenhagen
The Panum Institute Copenhagen, Denmark

Potassium permeability of the mesothelium of the frog mesentery

By

J Frokjær Jensen and Ove Christensen

Received 13 June 1978

Abstract

Frokjær Jensen J and O Christensen *Potassium permeability of the mesothelium of frog mesentery* Acta physiol scand 1979 105 228–238

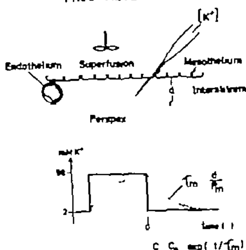
The mesothelium of the mesentery is a single cell layer described to offer no or very little barrier to diffusion of small water soluble solutes. We have measured the potassium permeability of the mesothelium of the frog mesentery both *in vivo* and *in vitro*. The permeability measured *in vivo*—using K$^+$-sensitive microelectrodes is 2.4 10^{-4} cm s^{-1}. *In vitro* measurements using conventional tracer flux technique on isolated mesentery yield a K$^+$-permeability of 5.7 10^{-4} cm s^{-1}. These values are 15–30 times smaller than values previously reported from *in vitro* experiments on rat and rabbit mesentery. Also, the permeability is 12 15 times lower than the K$^+$-permeability of the capillary wall determined on single capillaries in frog mesentery. In the frog mesentery the mesothelium thus represents an important diffusion barrier compared to the capillary wall. This may be critical in experiments where filtration and reflection coefficients of the capillaries are determined from measurements of fluid exchange across the capillary in response to application of hypertonic solutions on the surface of the mesentery

Many experimental studies of the filtration and diffusion properties of single capillaries have been performed on the capillaries of the frog mesentery (Landis and Sage 1971 Curry 1976, Crone *et al* 1978 Curry 1978) The frog mesentery is a very suitable preparation as microvessels here are larger than mammalian vessels and organized in a single layer of thin sheet of interstitial tissue The vessels are thus easily visible and accessible for experimental studies employing micro-tools.

The mesentery is covered by a single cell-layer the mesothelium. The presence of this layer is usually neglected in experimental studies of filtration- and diffusion properties of the capillary wall to water and to small ions. This is well justified if the resistance of the mesothelium to movement of ions and water is much smaller than the resistance of the capillary wall. *In vitro* measurements of the mesothelial permeability in rat and rabbit have largely confirmed this assumption (Rasio 1974 Berndt and Cosselin 1962). However studies of transmembrane ion transport through the autoperfused cat mesentery contradict the assumption (Frasher and Marcus 1976)

In this paper we report measurements of the permeability of the mesothelium of the frog mesentery to potassium ions. Measurements have been performed *in vivo* using

Schematic cross-section of the frog mesentery. The interstitium consists of an approx. 10 ... thick layer containing few cells but ... lot of fibrils. The interstitium embeds the capillaries whose walls are made up of endothelial ... The interstitium is covered towards the abdominal cavity by a single layer of cells forming ... mesothelium. The potassium concentration in ... interstitium is measured by ... potassium-sensitive microelectrode. The lower half shows the ... idea behind the experiment. The superfusion solution (heavy line) is changed in ... square manner and the response in interstitial concentration (dashed line) is followed. The mesothelial permeability is derived from the exponential time-course of interstitial potassium concentration.



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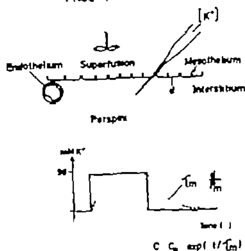
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The purpose of this paper is to stress the importance of the mesothelium as a diffusion barrier which can not be neglected in experiments on capillary fluid and solute exchange in frog mesentery.

Methods

Preparation

Experiments were carried out on *Rana temporaria* at room temperature (20–24°C). The animals had been kept at 4°C for varying periods up to 6 months. Anesthesia was established by placing the frogs either in 5 urethane or 0.5 MS 222 (tricaine methanesulfonate, Sandoz) solution until the mouth respiration stopped. The abdomen was opened ventrolaterally by electrocautery to minimize bleeding. The frog was then placed on its back on a small table and the mesentery carefully spread out over a small perspex window. It could be transilluminated and viewed through a Wild M5 stereo microscope. For positioning of potassium-sensitive microelectrodes and of perfusion pipettes, Leitz micromanipulators were used. The exposed mesentery was kept moist by a continuous superfusion with frog Ringer's solution.

Experimental technique

The basic idea behind the measurements of the mesothelial permeability is shown in Fig. 1. A potassium-sensitive microelectrode is inserted into the interstitium to measure the response in potassium concentration resulting from an abrupt increase of potassium concentration in the superfusion solution. When the concentration is raised abruptly from 2 mM to a value of 50 mM, the interstitial concentration will approach 50 mM in an exponential fashion. Alternatively if the potassium concentration in the superfusion solution is changed back to 2 mM again after the interstitial concentration has reached 50 mM, the interstitial concentration (c_i) will decline exponentially according to the relation

$$c_i = c_0 \exp(-t/\tau_m) \tag{1}$$

where c_s is the superfusion concentration and where τ_m is the decay time constant. Concentrations in the formula are changes in concentration above the physiological concentration of 2 mM, such that c_s is 48 mM

In this simple two-compartment system, where exchange of potassium takes place through the mesothelial surface the mesothelial permeability is given by the expression

$$P_m = d/\tau_m$$

where d is the thickness of the mesentery A mathematical analysis of the system is given in the Appendix.

In practice it proved to be rather difficult to change the superfusion concentration without disturbing the electrodes in position. Therefore another version of the experiment was adopted in order to load the interstitium with a high potassium concentration from a single perfused capillary. A perfusion pipette connected to a manometer is filled with an isotonic frog Ringer's solution containing [illegible] mM potassium. A capillary is cannulated with the pipette and continuously perfused by applying pressure to the pipette The superfusion concentration is maintained at 2 mM Potassium ions permeate the capillary wall and diffuse into the interstitium where they permeate the mesothelium to be lost in the superfusate. After some time a steady state situation is reached (cf Appendix). When steady state has been established the perfusion is stopped and the decay of interstitial potassium concentration towards the superfusate concentration is monitored by a potassium-sensitive electrode placed in the interstitium. After a short diffusional equilibration of interstitial potassium concentration the decay is monoexponential and allows determination of the decay time constant τ_m from which the permeability can be derived as described above The change from the filling up situation to a discharge situation is simply done by turning the stopcock to unload pressure [illegible] the perfusion pipette without changing the flow of superfusate. In this way mechanical interference with measurements is avoided Furthermore this method has the advantage of a steep concentration gradient across the mesothelium from the very beginning of the discharge period.

In vitro control experiments

The mesentery of *Rana temporaria* was mounted in an Ussing chamber with an area of 0.33 cm². [illegible] was added to the frog Ringer's solution at one side and samples were taken from the other side at intervals for 1 h. Unstirred layer effects were minimized by constant stirring on both sides by use of magnetic rods. Samples were counted by standard liquid scintillation technique and corrected for decay of the isotope. Several samples were taken from the 'hot' side during the experiment and counting of these showed that the ^{42}K-concentration on the 'hot' side remained constant during the experiment. Under these circumstances, the mesothelial permeability can be calculated from the equation

$$P_m = \frac{2V}{S} \frac{d(CPM)}{dt} \frac{1}{CPM}$$

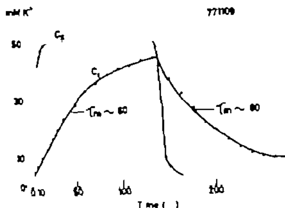
where V is the volume of one chamber (5 ml), S is the surface of mesentery (0.33 cm²), d(CPM)/dt is the rate of change of counts on the 'cold' side and CPM is the counts on the 'hot' side. The thickness of unstirred layers can be estimated from the time course of change in membrane potential, when 50 mM KCl is added to the medium on one side of the mesentery (Dainty 1966). The total thickness of the unstirred layers was estimated to be about 160 μm. The unstirred layers contribute thus to the calculated permeability with less than 5%.

The trans-mesenteric electrical resistance was measured at regular intervals during the experiment. The mean electrical resistance of the mesentery was 55 Ωcm ± 15 (S.D n = 5). Often we saw a decline in resistance of 20–40% within 1 h after mounting the mesentery This could indicate some minor deterioration with time due to the *in vitro* conditions. Contrary to this there was no indication of a systematic increase in the potassium permeability in the flux experiments. The counts on the 'cold' side increased linearly with time.

Miscellaneous

Microtools: The preparation of the necessary microtools (perfusion pipettes, potassium sensitive and holding pipettes) and a description of the electrical set-up has been given elsewhere (Crone et al.). Each electrode was calibrated separately

Isotopes. ^{42}KCl was obtained from AEK Risoe Denmark.



:xperimental result showing the response of interstitial potassium concentration in response to an change in superfusion concentration from 2 mM K to 50 mM K and back again. c_s is the response roelectrode just at the surface of the mesothelium and c_i is the simultaneous response of an electrode into the interstitium. The time constant for both the wash-in and the wash-out of interstitial m concentration 60 s.

ser Normal frog Ringer' solution was prepared to provide the following concentrations (mM): 107 K 2.0 Ca^{++} 1.8 Cl^- 116.4 HCO_3 1.2. The osmolarity was 215 mosmol/l. The *perfusate* consisted of normal frog Ringer with increased K -concentration, obtained by exchanging Na The potassium concentration was usually 50 mM. Bovine serum albumin (Sigma no. A-4503) was to final concentration of 1.25 . The *superfusate solution* consisted of normal frog Ringer with serum albumin added to concentration of 1.25 . In experiments ith increased K -concentra- NaCl was exchanged for KCl. *Calibration solutions* were normal frog Ringer' solutions with NaCl ged for KCl to give K -concentrations of 2, 4, 8, 16, 25, 50, 75 and 100 mM.
surements *of distance*: The distances were measured by use of an eyepiece micrometer with an ac of 5 μm.

Experimental results

re results

2 gives an example of an experiment, where the mesothelial permeability was determined the time course of interstitial potassium concentration (c_i) in response to an abrupt ge in superfusate concentration (c_s) from 2 mM K to 50 mM K and back again. concentration change in the superfusate is seen to be achieved within about 10 s while change in interstitial concentration is significantly retarded. The rise and decline of rstitial potassium concentration were characterised by exponential time courses with same time constant, from which the permeability was determined according to equa- (2).

ig. 3 gives an example of the other version of the experiment, where the interstitium is led with potassium from a continuously perfused capillary. When steady state was ched, the perfusion was stopped and the decay of interstitial potassium concentration recorded. The decay at different distances from the capillary within the mesentery was ained in consecutive runs. It is seen that after an initial time lag due to diffusional equili- tion in the interstitium, the curves at all distances show a monoexponential decline. The iation from the monoexponential time course is most marked close to the capillary

Fig. 3. Wash-out of potassium from the [illegible] compartment. The decay of interstitial [illegible] concentration is shown at various distances [illegible] continuously perfused capillary through [illegible] interstitium has been loaded with potassium [illegible] curve starts when the perfusion is stopped [illegible] curves are displaced for graphical reasons [illegible] experimental points and continuous curves [illegible] culated according to the formula in the appendix [illegible] $D_i = 2.6 \cdot 10^{-6}$ cm² s⁻¹ P = 35 10^{-3} cm [illegible] 65 s. The dashed line has a slope corresponding [illegible] decay time of 65 s. The experimentally [illegible] decay time from the curve at 120 µm is 55 s

where the initial concentration gradient is largest On the figure is also shown a fit [illegible] experimental data based on the theory given in the appendix. It is sufficient to state [illegible] that it can be shown that a good approximation to the true time constant for [illegible] of potassium can be obtained by determining the decay time constant from the slope [illegible] large times, *i.e* at times comparable to the decay time constant. The mesothelial permeability was again determined from equation (2)

The results from experiments on 12 frogs is given in Table I The mean thickness of [illegible] interstitial compartment was found from 44 cross sections of frog mesenteries prepared [illegible] electron microscopy An estimate of shrinkage of the tissue due to dehydration and [illegible] bedding was made by comparing the dimension of *in vivo* red blood corpuscles with [illegible]

TABLE I In vivo measurements of mesothelial decay time constants. The type of experiment is [illegible] S for superfusion experiments and P for perfusion experiments. For the superfusion experiments n is the number of determinations on the same position in the mesentery For the perfusion experiments, n is the number of different positions from the perfused capillary used in the determination.

Type of experiment	Frog	Decay time constant (s)	n
S	1	45	3
S	2	60	2
S	3	54	1
P	4	51	3
P	5	47	3
P	6	44	3
P	7	32	3
P	8	48	2
P	9	41	2
P	10	38	2
P	11	66	2
P	12	93	4
Mean value		51 6 ± 16 0 (s.d)	

l ones. A shrinkage of about 20% was found—a value in accordance with normally :d shrinkage due to preparation for E.M. When a correction for a shrinkage of this tude was applied the mean thickness, d, of the frog mesentery was found to be

$$12.2 \pm 4.0\ \mu m \quad (s.d.).$$

this figure is combined with the average decay time constant of 51.6 s (Table I) an mesothelial permeability P_m, of

$$2.4 \quad 10^{-4}\ cm\ s^{-1}$$

alculated. It is not possible to give the standard deviation on this value since correing values of thickness and decay time constant could not be determined on the same tery. The standard deviation on the decay time constant and the thickness are almost r (31% and 33%, respectively). The standard deviation would thus vary from about o 0% depending upon the degree of correlation between the decay time constant and ickness of the mesentery.

ro results

results from 5 control experiments using radioactive tracer technique on the *in vitro* aration gave a value of the mesothelial permeability of

$$P_m \quad 5.7 \pm 1.4\ (s.d.)\ 10^{-4}\ cm\ s^{-1}$$

ad expected a large discrepancy between mesothelial permeabilities based on *in vivo*- *in vitro*-techniques due to the possible shedding of the mesothelium of the *in vitro*-aration reported by Teel *et al.* 1971. Since this is not the case we have deferred from a e extensive investigation of the *in vitro* permeability.

re control experiments

questions concerning the *in vivo* experiments may be asked. The first concerns the ciency with which the superfusion solution clears the upper mesothelial surface of the ntery for potassium in excess of the concentration in the superfusate of 2 mM K. evaluation of the mesothelial permeability is for both experimental versions based on assumption of a superfusion concentration of 2 mM K.

response to this question, the experiment on Fig. 4 illustrates the efficiency of the rfusion in clearing any excess potassium and shows qualitatively the importance of the othelium as diffusion barrier.

single capillary is cannulated with perfusion pipette through which the capillary is tinuously perfused with a Ringer's solution containing 50 mM K. Further downstream otassium-sensitive electrode is positioned at various locations indicated in the inset of 4. First the electrode is inserted close t the capillary in the interstitium and the per on is started (A). Within 3 s the interstitial concentration t this place reach a value of ut 17 mM K. The electrode is withdrawn to a position just above the mesothelium in superfusate (S), inserted again (B) withdrawn, inserted (C) and withdrawn. In (D) the trode is positioned at the top of the perfused capillary and inserted in the interstitium

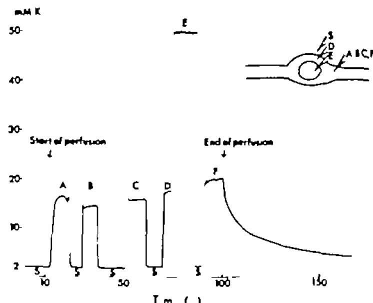


Fig. 4. Potassium concentr different positions in sup interstitium and capillary in response to perfusion of ... with 50 mM K ... solution. The K⁺-concentr. the superfusate is 2 mM. The ... sensitive microelectrode ... to the positions shown in the ... on the figure. For further ... refer to the text.

between the mesothelium and the capillary endothelium. A small tap at the micromanipulator brings the electrode tip into the capillary lumen (E) In (F) the electrode is ... in the interstitium close to the capillary again and the perfusion is stopped.

The first part of the figure, S to A shows how rapidly K diffuses across the capillary endothelium in comparison with the rather slow exchange of potassium across the mesothelium (Fig. 2) The experiment stresses the importance of the mesothelium as a diffusion barrier which in the steady state situation *i.e* after 50–100 s holds a concentration difference of 2–20 mM K (S to F) The figure illustrates how efficient the superfusion is in keeping the K -concentration constant at 2 mM K^+ on the surface of the mesothelium, even during repuncture of both mesothelium and the capillary wall Notice that the electrode responds immediately when it is moved from superfusate to interstitium and from interstitium to capillary which shows that there were no unstirred layers present

The second question about the *in vivo* experiments concerns the possible existence of a fluid layer under the mesentery between the lower mesothelial layer and the surface of the perspex rod Such a fluid layer would effectively increase the thickness of the mesentery, lead to a too large decay time constant and thus to a too small value of the mesothelial permeability Analysis of such a three-compartment system showed that if a fluid layer ... μm thick is present under a mesentery 10 μm thick the observed decay time constant will be larger by a factor of two than the true decay time constant. Consequently the calculated permeability would be underestimated by a factor of two

In order to estimate the thickness of the possible fluid layer under the mesentery 3 mesenteries were consecutively mounted under a light microscope (Leitz Laborlux, objective 3210 40) with high magnification (320 times) At this magnification the focal depth is ... 5 μm. The microscope was focused on a point in the tissue and a small glass rod (a ... pipette) was used to press the tissue down towards the perspex rod It was ... depress the tissue out of focus indicating the thickness of a

5 μm The calculated permeability is therefore u

it is worth mentioning that 68% of diffusional equilibration over an interstitial of 10 μm takes place in less than 0.1 s, assuming a value for the diffusion constant ium in the interstitium of 0.6 · 10^{-5} cm² s (Crone et al. 1978). rstitium can therefore be considered a well mixed compartment in the direction cular to the surface of the mesentery.

Discussion

ivo- and the in vitro-results give upper and lower boundaries for the permeability esothelium. The in vivo results may be underestimated by maximally a factor of two he possible existence of a fluid layer between the mesentery and the perspex rod. The esults therefore represent a lower limit of permeability. Contrary to this, the in vitro ments probably represent overestimates due to the in vitro conditions. The in vitro herefore represent an upper limit of permeability. The true mesothelial permeability likely to be found within the range of these values, i.e.

$$2.5 \cdot 10^{-5} < P_m < 5.7 \cdot 10^{-5} \text{ cm s}^{-1}$$

curacy of the in vivo and in vitro results are not good enough to postulate a real dif between in vivo- and in vitro-permeability although the permeability measured in vivo ler than the in vitro permeability.

value for the mesothelial permeability can be compared with other published values. n and Berndt (1962) determined the Rb-permeability of the in vitro rabbit mesentery · 10^{-5} cm s⁻¹. *The Rb-ion has approximately the same diffusion coefficient in a ns solutions as the potassium ion.* Rasio (1974) reported values of 79 · 10^{-5} cm s⁻¹ for otassium permeability of the rat mesentery in vitro. Fraser and Marcus (1976) ed a potassium permeability of 6.4 · 10^{-} cm s⁻ for the auto-perfused cat mesentery. ry (1978) has suggested, that albumin could affect the mesothelial permeability. In rate series of in vitro experiments, we tested this hypothesis by measuring the trans-teric electrical resistance. The presence of albumin in the Ringer's solution had no on the transmesenteric resistance.

e value of mesothelial permeability reported here is 12–25 times smaller than the potas- permeability of the capillary wall of 67 · 10^{-5} cm s⁻¹ (Crone et al. 1978). It thus re nts a poor model of the capillary wall, in contrast to the proposal by Rasio (1974). conclude, that in the frog, the mesothelium of the mesentery differs significantly in sional properties from the endothelium of the capillary. It represents an important sion barrier—a fact which may be crucial for the interpretation of experiments on otic fluid movement across the capillary wall induced by application of hypertonic tions on the surface of the mesentery (Landis and Sage 1971. Curry et al. 1976, Curry). The measured filtration of water across the capillary wall in these experiments is a tion of both capillary and mesothelial permeability and of geometrical factors as well.

thank professor C. rone for his continuous support during the course of this work. The authors are ful to Dr N. Bendixen for valuable help and laboratory facilities for the in vitro experiments. The Association is thanked for financial support.

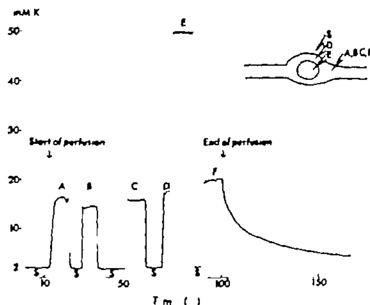


Fig. 4. Potassium concentration at different positions in superfusate, interstitium and capillary in response to perfusion of a capillary with 50 mM K -Ringer solution. The K⁺-concentration in the superfusate is 2 mM. The sensitive microelectrode is moved to the positions shown in the figure. For further details refer to the text

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The first part of the figure, S to A, shows how rapidly K diffuses across the endothelium in comparison with the rather slow exchange of potassium across the mesothelium (Fig. 2). The experiment stresses the importance of the mesothelium as a barrier which in the steady state situation, *i.e* after 50–100 s holds a concentration difference of 2–20 mM K (S to F) The figure illustrates how efficient the superfusion is in keeping the K -concentration constant at 2 mM K on the surface of the mesothelium, even after repuncture of both mesothelium and the capillary wall Notice that the electrode responds immediately when it is moved from superfusate to interstitium and from interstitium to capillary which shows that there were no unstirred layers present.

The second question about the *in vivo* experiments concerns the possible existence of a fluid layer under the mesentery between the lower mesothelial layer and the surface of the perspex rod Such a fluid layer would effectively increase the thickness of the mesentery and lead to a too large decay time constant and thus to a too small value of the permeability Analysis of such a three-compartment system showed that if a fluid layer μm thick is present under a mesentery 10 μm thick, the observed decay time constant will be larger by a factor of two than the true decay time constant. Consequently the calculated permeability would be underestimated by a factor of two

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$$2.5 \quad 10^{-6} < P_m \quad 5.7 \quad 10^{-6} \text{ cm s}^{-}$$

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Appendix

In this appendix we shall derive an expression for the time course of the decay of interstitial p concentration from the steady state in the situation where the interstitium is loaded through perfusion of the capillary When the capillary is perfused with a constant potassium concentr will penetrate the capillary wall and be transported out in the interstitium by diffusion. However, mesentery is flushed by a superfusion solution with a potassium concentration equal to the pl pot ssium concentration, ions will escape through the upper mesothelial layer As we have ea (Crone et al 1978) this leads to a steady state situation characterized by a spatial variation in p concentration given by

$$c_i(x) = c_i(0)\exp(-x/\lambda)$$

where $c_i(0)$ and λ are given by the expressions (cf Table A 1)

$$\lambda = (D_i\tau_m)^{\frac{1}{2}} \quad \tau_m = d/P_m$$

$$c_i(0) = \frac{c}{1+(D_i/\lambda P)}$$

This steady state concentration is sketched in Fig. A 1 Note that the capillary permeability is only ing in determining the value $c_i(0)$ of concentration at the capillary wall. The physiological p concentration equal to the one in the superfusion solution, has been subtracted from the conc given by Eq (A1) since only increme ts bove this value is being dealt with.

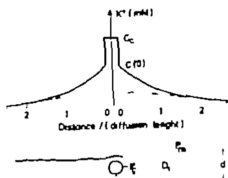
In order t describe the decay of the conce tration from the steady state hen the perfusion s some appro imations are appropriate We shall here neglect the contribution from the pota residing in the capillary The time scale relevant fo the decay situation is about 50 s. We have earlier that equilibration of the concentration difference across the capillary wall takes place during le so that in the prese t analysis, the presence of the capillary can be neglected. The potassium io in the capillary are neglected because they will redistribute in a volume much larger than the volume since the characteristic length for the interstitial concentration is about 200 µm. The effect time course of the decay from the steady state will therefore only be substantial close to the capillar

The cha ge of concentration in the interstitium is described by the partial differential equation (et al. 1978)

$$\frac{\partial c_i}{\partial t} = D_i\frac{\partial^2 c_i}{\partial x} - \frac{c_i}{\tau_m}$$

TABLE A 1 Symbols and units

Symbol	Unit	Significance
$c_i(x, t)$	mM	interstitial potassium ion con contration.
c_e	mM	capillary concentration.
$c_i(0)$	mM	i terstitial concentration at the capillary wall.
d	cm	thickness of the mesentery
x	cm	dist nce from capillary wall t current position i interstitium.
λ	cm	characteristic diffusion length withi the mesentery ($=(D_i\ _m)^{\frac{1}{2}}$).
P	cm s^{-1}	capillary permeability
P_m	—	mesothelial permeability
D_i	cm^2 s^{-}	diffusion const t f potassium ions in interstitium.
τ_m	s	decay time constant for transport through mesothelium ($=d/P_m$).



Sketch of potassium ion concentration in tissue versus distance normalized with re- the diffusion length. The full line shows the … concentration at time zero. The dashed … the calculated concentration at … times of the decay time constant τ_m. The lower half … shows the position of the capillary in the …

…ust be solved in space and time together …th the boundary condition at zero time:

$$c_i(0, \ ) \quad c_i(0) \exp(-| \ |/\lambda)$$

…he symmetry of this curve around the origo we obtain an additional boundary condition, that the … flux should be zero at the origo for times larger than zero

$$\left.\frac{\delta c_i}{\delta x}\right|_{0} = 0 \text{ for } t > 0$$

…quations can be solved using the technique of Laplace transformation. The results for the decay of …ial concentration from the steady stat…ls.

$$c_i(x, t) \quad c_i(0) [e^{|x|/\lambda} - \tfrac{1}{2}\{ \ e^{|x|/\lambda} \operatorname{erfc}(\ |/(2\sqrt{D}\ ) - \sqrt{t/\tau_m}) - e^{|x|/\lambda} \operatorname{erfc}(\ |/(2\sqrt{D\ t}) + \sqrt{t/\tau_m})\}] \qquad (A3)$$

…mple of this solution … shown in Fig. A 1 …. distance at … time of 0.46 τ_m after the end of perfusion. …usion process in the interstitium tends t… …ash out concentration differences. When this spatial …tion has taken place in … given region of space, the decay of concentration in this region is ap…ately exponential. This result can be obtained directly from Eq. (A2) when after sufficiently long …e diffusion term can be neglected.

…g. 3 … shows … fit of … to experimental data using the theoretical result in Eq. (A3). The values of …and τ_m were first derived from the steady state results as described by Crone et al. 1978 and next changed to obtain the best fit.

…rimentally we have determined the value of τ_m from the decay of concentration far away from the …ry where diffusion effects are of minor importance. The decay here is t… … good approximation …xponential. Nevertheless, this method tends to underestimate the value of τ_m as seen in Fig. 3. …tion to derive … value for τ_m from … plot of the theoretical result (A3), using the slope of the decay …at … time equal to τ_m the value thus derived is 10–15 … smaller than the value used in the calculation. …ore, the experimentally determined value of τ_m should be underestimated by the same amount.

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From the Department of Physiology, University of Göteborg, Sweden

Lack of specific prostaglandin antagonistic effects of 7-oxa-13-prostynoic acid on ovarian metabolism *in vitro*

By

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Abstract

Magnusson, C., G. Selstam and K. Ahrén. *Lack of specific prostaglandin antagonistic effects of 7-oxa-13 prostynoic acid on ovarian metabolism in vitro*. Acta physiol. scand. 1979. *105*. 239–247.

[illegible]aglandins (PGs) have been proposed to function as obligatory intermediates in the action of luteinizing [illegible]mone (LH) (Kuehl *et al.* 1970). This was based on experiments on isolated mouse ovaries where the [illegible] 7-oxa-13-prostynoic acid (7-oxa-13-PA) was found to block the effect of both PGE_1 and LH [illegible] cyclic AMP formation and progesterone synthesis. W[e] have studied [w]hether 7-oxa-13-PA acts [illegible] PG antagonist also on the isolated rat ovary where PGs of the E type have many effects. Prepubertal [illegible] were incubated up to 4 h in modified Krebs bicarbonate buffer containing glucose with PGE_1 [illegible] 7-oxa-13-PA, alone and in combination. PGE_1 stimulated the uptake of the non-utilizable amino acids [illegible]butyric acid (AIB) and cycloleucine in an equally wide dose-range as has earlier been found for [illegible]ulation of lactic acid and cyclic AMP production as well as protein synthesis. 7-oxa-13-PA alone, [illegible]centrations exceeding 25 µg/ml, stimulated lactic acid production, but inhibited the incorporation [illegible]belled leucine and the uptake of AIB. The uptake of cycloleucine was slightly stimulated. 7-oxa-13-PA [illegible] not antagonize the PGE_1 effect on lactic acid production. 7-oxa-13-PA diminished the PGE_1 effect on [illegible] uptake of amino acids and the incorporation into protein, but only in concentrations where it in itself [illegible]ited the protein synthesis. These results show that 7-oxa-13-PA does not act as [illegible] PG-antagonist [illegible] the prepubertal rat ovary [illegible]ause it cannot block the effects of PGs without having marked inherent [illegible]ts.

[illegible]nsive work is being done in order to elucidate the role of prostaglandins (PGs) in the [illegible]chanism of action of various hormones. For this purpose several types of inhibitors of [th]e action of PGs have been developed (for ref. see Sanner 1974). In the late sixties Fried [an]d co-workers synthesized a group of substances with close structural similarity to PGs [(Fr]ied *et al.* 1969). Several of these 7-oxa-prostaglandin analogues inhibited PG-induced [con]tractions of isolated intestinal smooth muscle preparations in a competitive manner. [T]he most specific PG inhibitors were the analogues with a triple bond in the 13, 14-position. [Oth]er workers, however, did not get the same clear-cut results. Fleck (1970) considered 7-[oxa]-13-prostynoic acid (7-oxa-13-PA) as the only specific PG inhibitor among these ana-[logues]. In several other experimental systems even this substance had no inhibitory effect.

In 1971 Bennett and Posner reported that 7-oxa 13-PA did not specifically inhibit the K-induced contractions of several different intestinal smooth muscle preparations. In spite of these contradictory findings, 7-oxa 13-PA has been widely used as a PG antagonist in several different experimental systems (see *e.g.* Sanner 1974).

PGs exert many different actions in the reproductive sphere. The E type has been shown to mimic many effects of gonadotropins on ovarian metabolism. Thus, in the ovary they stimulate steroid synthesis (Pharriss *et al.* 1968, Marsh 1970, Speroff and Ramwell 1970), formation of cyclic 3',5' adenosine monophosphate (cAMP) (Kuehl *et al.* 1970, Marsh 1971, Ahrén *et al.* 1974, Selstam *et al.* 1974), glycolysis (Perklev and Ahrén 1971) and the amino acid transport and incorporation into protein (Ahrén and Perklev 1973). It was therefore of great interest when Kuehl and co-workers (1970) reported that 7-oxa 13-PA (50-75 μg) inhibited not only the PG-induced cAMP formation in the mouse ovary but also the stimulatory effect of LH on the cAMP formation. In contrast to these findings no inhibition of the stimulatory effect of LH or PGs on cAMP formation was found in porcine granulosa cells (Kolena and Channing 1972) or rat ovaries (Lamprecht *et al.* 1973). Ellsworth and Armstrong (1974) could not find that 7-oxa 13-PA affected the luteinization of granulosa cells in transplanted rat ovarian follicles in response to LH or PGE_2. Channing (1972) could find no an inhibition on luteinization of monkey granulosa cells, but when added together with human chorionic gonadotrophin (HCG) or PGE_2, 7-oxa 13-PA produced necrotic changes in the cells.

In the light of these studies it was considered to be valuable to study more in detail if 7-oxa 13 PA has PG-agonistic and/or PG-antagonistic action. The effects of 7-oxa-13-PA alone and in combination with a prostaglandin, on the carbohydrate and amino acid metabolism were studied in an experimental system often used in our laboratory, the prepubertal rat ovary. Some preliminary results have been reported earlier (Ahrén and Perklev 1973).

Materials and Methods

Animals

Rats, 23 days old, of the Sprague Dawley strain, were used. A standard pellet diet was given and water *ad libitum*. The rats were obtained from Anticimex Ltd, Stockholm and deprived of food 18-24 h before the experiment.

Chemicals and hormones

Generally tritiated α-aminoisobutyric acid (AIB-³H), AIB-1-¹⁴C, 1-aminocyclopentane-1-carboxylic acid-¹⁴C (cycloleucine-¹⁴C), generally labelled leucine-³H and leucine-¹⁴C, and tritiated cyclic 3',5'-adenosine monophosphate (³H-cAMP) were obtained from New England Nuclear Co., Boston, Mass., USA. The non-utilizable amino acids AIB and cycloleucine had a final concentration in the incubation medium of 0.1 mM. AIB-³H had an activity of 0.33–1.65 μCi/ml, AIB-¹⁴C 0.057–0.2 μCi/ml and cycloleucine 0.025–0.1 μCi/ml. The labelled natural amino acids leucine-³H and leucine-¹⁴C were used in a final concentration of 0.01 mM and had an activity of 1.98 μCi/ml and 0.2 μCi/ml, respectively. cAMP dependent protein kinase and protein kinase inhibitor for the cAMP assay were obtained from Sigma Ltd. All other chemicals were of analytical grade and purchased from Sigma Ltd and Merck Co.

PGE and PGE_1 were supplied by Ono Pharmaceutical Co. Ltd, Osaka, Japan and $PGF_{2\alpha}$ by Upjohn Co. The 7-oxa 13-PA was kindly given to us by Prof. J. Fried, Dept. of Chemistry and Biochemistry, Univ. of Chicago, Ill., USA. The PGs and the 7-oxa 13-PA were dissolved in ethanol (1 μl ethanol per μg PG and 0.2 μl per μg 7-oxa 13-PA). Aliquots of these solutions were added directly to the incubation medium. Because ethanol in concentrations exceeding 10 μg/ml incubation medium in itself significantly inhibits

…cid production and increased cycloleucine uptake, concentrations higher than 20 µg PGE_1/ml or …oxa-13-PA/ml were not tested. Bovine LH (NIH-LH-B8) was generously supplied by the Endocrino…logy Section of the National Institutes of Health, Bethesda, Md, USA.

…ental procedure

…were killed by cervical fracture and the ovaries were rapidly removed and chilled in buffer. They …ected free of extraneous tissues under microscope, rinsed, blotted on filter paper and each pair of … put into … 10 ml Erlenmeyer flask. The ovaries were incubated at 37°C for 30–240 min in 1 ml …l Krebs-Ringer bicarbonate buffer (1.25 mM $CaCl_2$) pH 7.4 equilibrated with 95 … O_2 + 5% CO_2, …dition of 1 mg glucose/ml. The labelled substances, the PGs and 7-oxa-13-PA were added in dif… combinations as indicated in Results.

… incubation the ovaries were rinsed, weighed, and homogenized in 10 … trichloroacetic acid (TCA) …rifuged. Aliquots from the supernatant and incubation media were taken for radioactivity deter… …as according to Nilsson and Selstam (1974). The uptake of the labelled amino acids is given as the …tion ratio at the end of the incubation period, calculated according to Ahrén and Rubinstein (1965). …TCA precipitates were used for determination of the incorporation of radioactivity into protein as …ed earlier (Nilsson and Selstam 1974) and expressed as DPM/µg protein. The protein content was …ned according to Lowry *et al.* (1951).

…c acid accumulation in the medium was determined at the end of the incubation period according …dlin *et al.* (1963 a, b). The tissue content of cAMP was determined according to Gilman (1970). …ntent of cAMP in the medium was determined by … modification of this procedure as has been …ed earlier (Selstam *et al.* 1974).

…tical procedure

…values are given … standard error of the means. All groups presented as figures or mentioned in the …present the mean of 5–15 pairs of ovaries. The values were compared by analysis of variance, followed …Student-Newman-Keul' … multiple range test for comparison between individual groups (Woolf 1968). …lity of less than 0.05 was considered significant.

… 1. Effects of different prostaglandins and fatty acids on lactic acid production and amino acid uptake …by prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicar…bonate buffer containing 5.5 mM glucose. The lactic acid was measured at the end of the incuba…tion period. The amino acid uptake is given as distribution ratio at the end of the incubation period.

…bstance …		Lactic acid production (µg/ml tissue)	AIB-³H distribution ratio (CPM/ml intracellular water / CPM/ml medium)
—			
/			
…ol		1.58 ± 0.17	14.8 ± 0.4
	0.1	2.37 ± 0.11**	18.8 ± 0.6**
	10	2.64 ± 0.17**	22.9 ± 0.5**
	0.1	2.49 ± 0.17*	20.4 ± 1.2**
	10	2.73 ± 0.17**	21.8 ± 1.2*
	0.1	1.48 ± 0.08 N.S.	16.1 ± 0.4 N.S.
	10	2.92 ± 0.15**	19.6 ± 1.2**
2			
…ol		1.15 ± 0.07	11.2 ± 0.4
	0.1	1.95 ± 0.09*	14.9 ± 1.0**
…idonic acid	0.1	1.07 ± 0.05 N.S.	10.5 ± 0.4 N.S.
	50	1.16 ± 0.07 N.S.	11.3 ± 0.3 N.S.
…ic acid	0.1	1.30 ± 0.06 N.S.	10.7 ± 0.3 N.S.
	50	0.63 ± 0.09*	11.3 ± 0.7 N.S.

…ls of significance vs. controls: … = $p < 0.01$ N.S. not significant.
*…

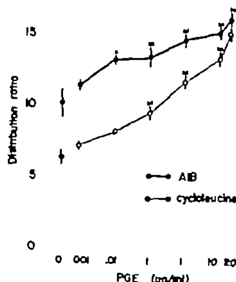


Fig. 1 Effect of PGE_1 on the uptake of AIB and cycloleucine in prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose, 0.1 mM AIB and 0.1 mM cycloleucine ^{14}C. The uptake of amino acids is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE_1 effect: $p < 0.05$, $p < 0.01$ N.S. = not significant.

Results

Effects of PGs

The effects of PGE, PGE_2 and $PGF_{2\alpha}$ on lactic acid production and uptake of AIB compared (Table I). The potency of PGE and PGE were equal, while PGF was potent. The uptake of cycloleucine was also measured after 2 h of incubation. PGE, P and $PGF_{2\alpha}$ showed effects similar to those on the AIB-uptake (not shown in the T. The PG precursor arachidonic acid and the fatty acid palmitic acid had no effect on any of the parameters (Table I).

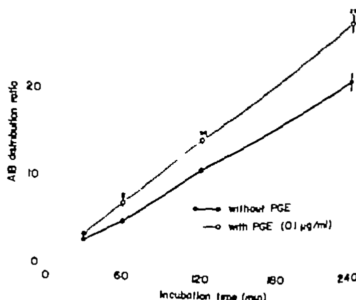
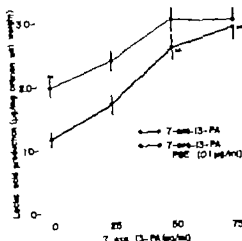


Fig. 2. Time-relationship of the uptake of AIB in prepubertal rat ovaries incubated with or without PGE (0.1 µg/ml). The ovaries were incubated in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB-^{14}C or AIB-H. The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE effect at the different times: p
N.S. = not significant.



Effect of 7-oxa-13-PA alone and in com[...] with PGE_1 (0.1 µg/ml) on the lactic acid [...]tion by prepubertal rat ovaries. The ovaries [...]ubated for 2 h in modified Krebs-Ringer [...]nate buffer containing 5.5 mM glucose. [...]c acid was measured at the end of the [...]ion period. Levels of significance: * = p < [...] ** p 0.01; N.S. not significant. Levels [...]ificance of the effect of the different con[...]tions of 7-oxa-13-PA alone are indicated at [...]er curve. Levels of significance of the effect [...]ded PGE_1 at the various concentrations of [...]13-PA are indicated at the upper curve.

[...]E, stimulates lactic acid production in a dose-dependent way in the concentration [...] 0.025–0.8 µg/ml (Perklev and Ahrén 1971). In the present study we found that PGE_1-[...]ntrations exceeding 0.8 µg/ml did not give any further stimulation of the lactic acid [...]ction (data not shown). PGE_1 also stimulated the uptak of the non-utilizable amino [...] AIB and cycloleucine in the ovary in the same range of concentrations that stimulated [...] acid production (Fig. 1). The time-relationship of the effect of PGE_1 (0.1 µg/ml) on [...]AIB-uptake is shown in Fig. 2. The uptake is linear up to 4 h with time both with and [...]out PGE_1.

[...] control ovaries, the *incorporation of leucine-¹⁴C into ovarian protein after 2 h of* [...]bation was 879 ± 22 DPM/µg protein and increased to 1 078 ± 42 DPM/µg protein [...] stimulated by 0.1 µg PGE_1/ml medium. No further increase in incorporation rate was [...] with higher PGE concentrations.

[...]cts of 7-oxa-13-PA

[...]a-13-PA in concentrations ranging from 25 to 75 µg/ml stimulated the lactic acid produc[...] in dose-related manner (Fig. 3), *i.e.* had an effect similar to that of PGE_1. The effect [...]dded PGE was not abolished by 25 µg 7-oxa-13-PA/ml. With higher concentrations of [...]a-13-PA, in itself strongly stimulating the lactic acid production, PGE_1 could not give [...] further increase. In contrast to the effect on lactic acid production, 7-oxa-13-PA de[...]sed the uptake of AIB, *i.e.* an effect opposite to that of PGE_1 (Fig. 4 a). The effect of [...]ed PGE_1 was not abolished by 7-oxa-13-PA. On cycloleucine-uptake, 7-oxa-13-PA had [...]imulatory effect (Fig. 4 b), but, as in the case of AIB-uptake, PGE could stimulate the [...]loleucine-uptake when 7-oxa-13-PA was present.

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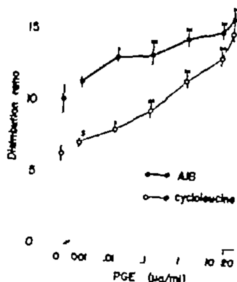


Fig. 1 Effect of PGE on the uptake of AIB and cycloleucine in prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose 0.1 mM AIB and 0.1 mM cycloleucine-^{14}C. The uptake of amino acids is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE effect: $p < 0.05$ $= p < 0.01$ N.S. = not significant

Results

Effects of PGs

The effects of PGE PGE and $PGF_{2\alpha}$ on lactic acid production and uptake of AIB compared (Table I) The potency of PGE_1 and PGE were equal while PGF was less potent The uptake of cycloleucine was also measured after 2 h of incubation. PGE_1, PGE and $PGF_{2\alpha}$ showed effects similar to those on the AIB-uptake (not shown in the Table). The PG precursor arachidonic acid and the fatty acid palmitic acid had no stimulatory effect on any of the parameters (Table I).

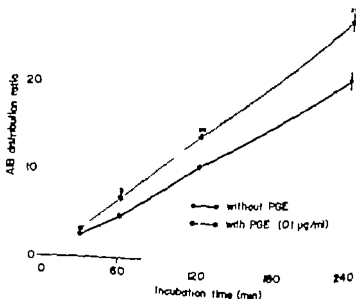
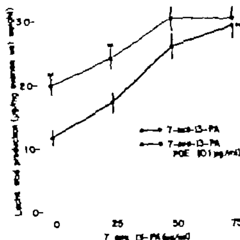


Fig. 2. Time-relationship of the uptake of AIB in prepubertal rat ovaries incubated with or without PGE (0.1 µg/ml). The ovaries were incubated in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB-^{14}C or AIB-^{3}H. The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE effect at the different times. N.S. = not significant.



Effect of 7-oxa-13-PA alone and in combination with PGE_1 (0.1 μg/ml) on the lactic acid production by prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose. Lactic acid was measured at the end of the incubation period. Levels of significance: * = $p < 0.01$, N.S. = not significant. Levels of significance of the effect of the different concentrations of 7-oxa-13-PA alone are indicated at the lower curve. Levels of significance of the effect of added PGE_1 at the various concentrations of 7-oxa-13-PA are indicated at the upper curve.

E stimulates lactic acid production in a dose-dependent way in the concentration 0.025–0.8 μg/ml (Perklev and Ahrén 1971). In the present study we found that PGE_1-concentrations exceeding 0.8 μg/ml did not give any further stimulation of the lactic acid production (data not shown). PGE_1 also stimulated the uptake of the non-utilizable amino AIB and cycloleucine in the ovary in the same range of concentrations that stimulated acid production (Fig. 1). The time-relationship of the effect of PGE (0.1 μg/ml) on AIB-uptake is shown in Fig. 2. The uptake is linear up to 4 h with time both with and out PGE_1.

control ovaries, the incorporation of leucine-^{14}C into ovarian protein after 2 h of incubation was 879 ± 22 DPM/μg protein and increased to 1 078 ± 42 DPM/μg protein stimulated by 0.1 μg PGE_1/ml medium. No further increase in incorporation rate was with higher PGE concentrations.

Effects of 7-oxa-13-PA

7-oxa-13-PA in concentrations ranging from 25 to 75 μg/ml stimulated the lactic acid production in a dose-related manner (Fig. 3), i.e. had an effect similar to that of PGE. The effect of added PGE_1 was not abolished by 25 μg 7-oxa-13-PA/ml. With higher concentrations of 7-oxa-13-PA, in itself strongly stimulating the lactic acid production, PGE_1 could not give further increase. In contrast to the effect on lactic acid production, 7-oxa-13-PA decreased the uptake of AIB, i.e. an effect opposite to that of PGE_1 (Fig. 4 a). The effect of added PGE_1 was not abolished by 7-oxa-13-PA. On cycloleucine-uptake, 7-oxa-13-PA had inhibitory effect (Fig. 4 b), but, as in the case of AIB-uptake, PGE_1 could stimulate the cycloleucine-uptake when 7-oxa-13-PA was present.

The protein synthesis was inhibited by 7-oxa-13-PA in a dose-dependent way. Again PGE had a stimulatory effect (Fig. 5), but only with 25 μg 7-oxa-13-PA/ml where it in itself did decrease the protein synthesis. Since 7-oxa-13-PA inhibited the protein synthesis it was compared to a specific inhibitor of the protein synthesis in the ovary. Puromycin in a dose (10 μg/ml incubation medium) which inhibits the incorporation of labelled natural amino acids approx. 95% (Ahrén and Rubinstein 1965), decreased the lactic acid production

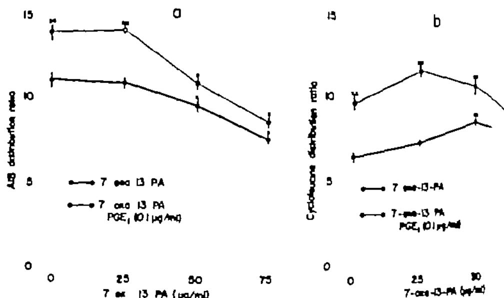


Fig. 4 a and b Effect of 7-oxa 13-PA alone and in combination with PGE$_1$ (0.1 μg/ml) on the uptake of AIB (a) and cycloleucine (b) in prepubertal rat ovaries. The ovaries were incubated for 2 h in Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB-^{3}H or 0.1 mM cycloleucine at 37 °C. The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance are indicated as in Fig. 3

(control 1 16 ± 0 14 puromycin 0.34 ± 0 06 μg/ml w w), somewhat decreased the AIB uptake (control 8.5 ± 0 7 puromycin 6 4 ± 0.3) and increased cycloleucine uptake (control 4.1 puromycin 6.7 ± 0.3) Thus, on amino acid uptake and protein synthesis, 7-oxa-13-PA acted very similar to puromycin

Due to the abovementioned marked inherent effects of 7-oxa 13-PA at concentrations above 25 μg/ml it was considered meaningful to test the effects of 7-oxa-13-PA on the cAMP system only at lower concentrations. 7-oxa 13-PA (1 and 20 μg/ml) did in itself not change the cAMP content in the tissue nor the release of cAMP into the incubation medium (Table II) When 1 μg 7-oxa 13-PA/ml was added in combination with a gonadotrophin

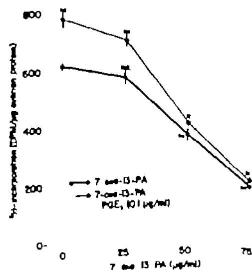


Fig. 5 Effect of 7-oxa 13-PA alone and in combination with PGE (0.1 μg/ml) on the incorporation of radioactivity from leucine-^{3}H into ovarian protein. Prepubertal rat ovaries were incubated for 2 h in Krebs-Ringer bicarbonate buffer containing glucose and 0.01 mM leucine-^{3}H Levels of significance are indicated as in Fig. 3.

i Influence of 7-oxa-13-PA on the stimulatory effect of LH on the cAMP content in the ovary and the incubation medium. The ovaries were incubated for 2 h in modified Krebs Ringer bicarbonate buffer containing 5.5 mM glucose.

-PA	cAMP (pmoles/mg protein)			
	control		NIH-LH-B8 (1 µg/ml)	
	tissue	medium	tissue	medium
	15 ± 1	N.D.	72 ± 8	350 ± 120
	14 ± 1 N.S.	N.D.	74 ± 10 N.S.	166 ± 32 N.S.
	20 ± 2 N.S.	N.D.	166 ± 27**	127 ± 36

not detectable.
of significance of the effect of 7-oxa-13-PA. * = p 0.05 ** = $p < 0.01$ N.S. = not significant.

ml), no effect was seen. At 20 µg/ml, however an increase in the tissue content with a temporaneous decrease in the release of cAMP was found.

Discussion

of the important questions in the reproductive field is what roles the different PGs may When studying the function of PGs, several different experimental approaches have used. Among these, we have further studied several gonadotropic effects that can be ...cked by the PGs, and we have also tested a proposed inhibitor 7-oxa-13-PA on these meters.

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...nother way of testing the effects of PGs is by the addition of antagonists. One obvious ...rion for an inhibitory substance is that the antagonist in inhibitory concentrations ...uld have no or at least a less pronounced effect than the agonist, when competing for receptor site. In this study the PG analogue 7-oxa-13-PA could not fulfil this criterion ...ould only inhibit the PGE-effects in concentrations where it in itself had a marked ...mulatory or inhibitory effect.

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In combination with LH, 7-oxa-13-PA exerted an effect on the cAMP system. The release ...cAMP from the ovary was decreased and the tissue content increased. This redistribution ...y be due to a non-specific effect on the membrane, since in these concentrations 7-oxa-...PA does not change adenylate cyclase activity as has been shown for many tissues by ...ls *et al.* (1975).

The question arises whether the different effects of 7-oxa-13-PA are dependent upon each

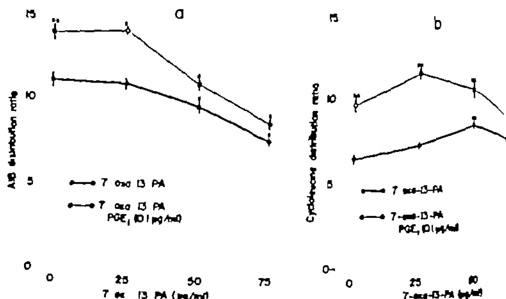


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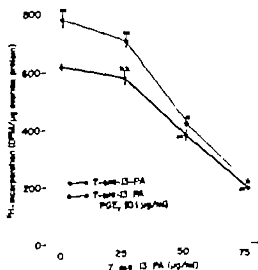


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N.D. = not detectable.
Levels of significance of the effect of 7-oxa-13-PA: * = $p < 0.05$, ** = p 0.01 N.S. = not significant.

...ml), no effect was seen. At 20 µg/ml, however an increase in the tissue content with a simultaneous decrease in the release of cAMP was found.

Discussion

One of the important questions in the reproductive field is what roles the different PGs may play. When studying the function of PGs, several different experimental approaches have been used. Among these, we have further studied several gonadotropic effects that can be mimicked by the PGs, and we have also tested a proposed inhibitor 7-oxa 13-PA on these parameters.

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The question arises whether the different effects of 7-oxa-13-PA are dependent upon each

other e.g are the effects secondary to a general impairment of membrane function or the inhibition seen in protein synthesis? As for the latter suggestion, inhibitors of synthesis can inhibit AIB transport (Ahrén and Rubinstein 1965) and stimulate cyc transport (Ahrén, Hillensjö and Selstam, unpublished), both findings confirmed in study It is therefore quite possible that the similar effects of 7-oxa 13-PA on acids might be secondary to the inhibited protein synthesis. On the other hand an membrane function may explain the change in amino acid transport as well as the in cAMP release and could perhaps also lead to an increased demand for energy as indicated by the increased lactic acid production

In order to study the role of PGs in the ovary however it is also necessary to whether PG-content and synthesis are changed after gonadotropic stimulation. In the and the ovary an increase of PGs has been shown after LH stimulation, but this increase registered *after* an increase in cAMP content (LeMaire *et al* 1973 Marsh *et al.* 1973, *et al* 1974) Inhibitors of PG synthetase, such as indomethacin and aspirin, are ineffec in inhibiting the LH effect on adenylate cyclase as discussed by Marsh (1975).

Another mode of procedure when testing a possible role for PGs in the gonadotr action is to study whether there is an additivity or not between the PG and the gona tropin Additivity would speak against a role of PG as an intermediate step in the gona tropin action, while a lack of additivity is an indication, but not evidence of a role of P Moreover in the ovary additivity studies are complicated by the many different cell ty which at least in part can be responsible for the different time-courses in cAMP respo after LH and PGE stimulation (Selstam *et al* 1974)

In view of the findings that PGs mimic many of the actions of gonadotropins and t PG effects are related to changes in the cAMP system, it has been tempting to postulat functional role for PGs in the ovarian metabolism Inhibitors of PG action are one import tool for studying this role. Our conclusion from the present study however is that 7-o 13-PA is not appropriate in this respect, since it does not act as a PG inhibitor in the ovar

The authors wish to thank Prof J Fried, Department of Chemistry and Biochemistry Universi Chicago, Ill., USA, for the gift of 7-oxa-13-PA and valuabl suggestions Thanks are also due to Leo Helsingborg, Sweden, fo the supply of PGs and to NIH for the supply of LH. The assistance given Stina Öberg, B.A., is gratefully acknowledged This work was supported by grants from the Swed Medical Research Council (B77 14X 27), M gnus Bergvall's Foundation, Wilhelm and Martina Lu gren s Foundation and from the Faculty of Medicine Göteborg.

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In vitro release of intestinal dipeptidases from everted rings of rat jejunum

By

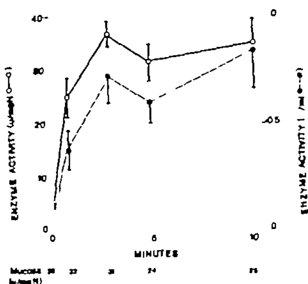
J JODL and T LINDBERG

Glycyl-leucine dipeptidase (EC 3 4 13.2) and proline dipeptidase (EC 3 4 13.9), shown localized in the cytoplasma of the enterocytes (Norén *et al.* 1977), were quickly rel. from *in vitro* preparation of pig small intestinal mucosa (Josefsson and Sjöström 19 About 50% of the total activity was released within 10–20 s from intact mucosa at slow magnet stirring. These dipeptidases were also easily released from similar prepar of rat jejunal mucosa *in vitro* (Lindberg *et al* 1975 Silk and Kim 1976). The contrast situation *in vivo* only small amounts of glycyl-leucine dipeptidase activity was loc small intestinal content in man (Josefsson *et al* 1968) and in rat (Josefsson and Lin in Lindberg *et al* 1975). There was no difference between the *in vivo* and *in vitro* rel proline dipeptidase

The present experiments were performed to determine the release of these dipep from everted rings of rat jejunum into the medium, employing an experimental t commonly used for *in vitro* absorption studies (*cf* Wilson 1962)

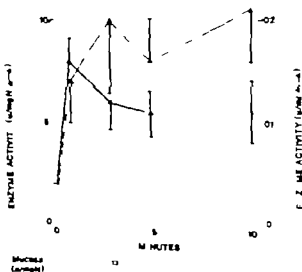
Methods Rats (Sprague-Dawley strain), 200 to 250 g, fasted for 12 h in metabolic cages with free to water Under ether anesthesia the small intestine was removed. Everted rings were prepared from jejunum (about 10 cm from duodenojejunal flexure). 6 rings were put in each incubation flask or 5 ml of Krebs-Ringer phosphat saline gased with O_2 and with 10 mmol/l glycyl-L-leucine (Sigma) 2 mmol/l L-alanyl-L proline (Sigma). Incubation was performed with shaking at 37°C under O_2.
At the appropriat time, 1 ml of incubation medium was removed, cooled to 4°C (freezing destroyed dipeptidase activity completely) and assayed immediately for dipeptidase activity according to Josefsson and Lindberg (1965). One of the everted rings was homogenized and analysed for dipeptidase activity. The enzyme assays were performed under optimum pH conditions (Josefsson and Lindberg 1965). unit of dipeptidase is the activity hydrolysing 1 µmol of dipeptide per min at 40°C.
Protein was determined according to Lowry *et al.* (1951) using crystallized bovine ribonuclease standard. The nitrogen content was calculated as 17 of the protein content.

Results Fig. 1 and 2 demonstrate the release of glycylleucine and of proline dipeptidase activities into the incubation medium. The activities are expressed as units per mg N per ml of medium The dipeptidase activities in the mucosa in the rings are given for parison Obviously both enzymes are quickly released considerable activity is found after 1 min The specific activity is as high as in the mucosa. 0.5 to 1.0 mmol/l glycyl-L-leucine and 0 1 to 0.2 mmol/l L-alanyl-L-proline are split per min by the enzymes released into the incubation medium



—Glycyl-leucine dipeptidase (substrate glycyl-L-leucine) ... during incubation with jejunal rings in the rat. ... activity expressed as ... N (O—O) and U/ml ... Mean values ± S.E. Mean values for mucosal ... dipeptidase activity ... for comparison.

...anus. The present study shows that a quick release of the cytoplasmic glycyl-leucine ...tidase and proline dipeptidase occurs from the mucosa into the incubation medium ... the *in vitro* experimental model of everted intestinal rings. Both this model and that ...rted intestinal sacs are the most commonly used in *in vitro* studies of the absorption of ...tides and amino acids (*cf.* Matthews 1975, Wiseman 1977). A considerable fraction ...ptide applied to the incubation medium can be hydrolysed within minutes by the ...ed enzymes. Glycyl-leucine dipeptidase has a wide specificity (Norén *et al.* 1973),



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In view of these findings it is important to consider these properties of the cytosolic intestinal dipeptidases when interpreting the results of dipeptide absorption studies *in vitro*

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...m the [1]Department of Physiology III, Karolinska Institutet and the [2]Department of Physiology II, Karolinska Institutet, Stockholm, Sweden

Control of the trunk during walking in the cat

By

[1]HANS CARLSON, JÖNT HALBERTSMA and MICHAEL ZOMLEFER

...ere is a great deal of information concerning the electromyographic (EMG) patterns ...vements of the hindlimb in tetrapod locomotion (Grillner 1975), there is little ...ge about the control of the trunk. Recent experiments performed on the cat's back muscles have shown large portions, / M. longissimus and iliocostalis, to be ...tracting and easily fatiguable, while the multifidi contract somewhat more slowly ... 1978 a, b). The aim of this study has been to explore patterns of activity in the ...t lumbar back muscles during fast and slow walks and relate them to the trunk ...ents.

...ca experiments were performed on cats (n 3 2.2, 2.3 and 3.2 kg), which were ...l to walk on a moving treadmill or overground at cycle durations ranging from about 1 200 ms, corresponding to speeds ranging from 1.0 to 0.2 m/s, respectively. Bipolar ...muscular electrodes were chronically implanted (Zomlefer *et al.* 1977) into the multi... ...M), longissimus (Lo) and iliocostalis muscles at various levels along the lumbar (L1–L6), as well as into both vasti lateralii (VL). The EMG wires were led per...ously to a plug on the animal's back, which could be connected with a detachable ... to amplifiers. Electrode placement was verified post mortem. The movements of one ...mb and the lower spine were recorded using a Selspot System (Selcom Co Partille, ...en). Small light emitting diodes were glued onto the shaved skin over the spinal ...ses at Th9 L3, and L5 as well as the iliac crest and the hip knee, ankle ...tarsophalangeal joints. Two cameras sampled each diode position at 160 Hz in both the ...al and horizontal planes. Position and electromyographic data were fed online to a ...computer (Hewlett-Packard 21MX) for later processing.

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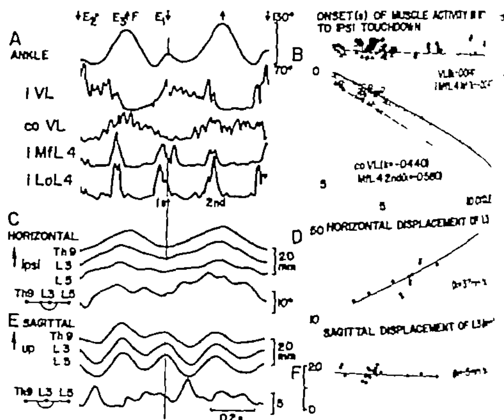


Fig. 1 A) Ankle angle (ANKLE) with rectified and filtered EMG's from ipsi- and contralateral VL as the ipsilateral Mf and Lo muscles at the L4 level (IVL, coVL, IMfL4 and ILoL4, respectively). for labeling, shown for the ipsilateral hindlimb, is the same as that used by Philippson (1905). B) Or EMG activity (relative to ipsilateral hindlimb touchdown) vs. cycle duration (CYCLE DUR) for IVL and the first and second bursts of IMfL4 (IMfL41st and IMfL42nd). A positive value (+) indicates onset time is prior to ipsi-hindlimb touchdown The time for onset of coVL will increase with cycle duration, since both hindlimbs are alternating in given step cycle. C) Horizontal plane displ. and angle. The three uppermost curves show the relative trajectories of the marker diodes at the L3 and L5 levels. Movement toward the ipsilateral side (see A) is defined by arrow (↑). The last shows the net angular displacement determined by L5–L3–Th9 (see inset). Minimum value to lateral bending with concavity toward the ipsilateral side. D) Peak to peak displacements in hor plane (L3 level) vs. step cycle duration E) Sagittal displacements and angle The first three traces the movements of the three back diodes (Th9 L3 and L5 levels) as seen from the ipsilateral side. ment upward is denoted by arrow (↑). I the three cats investigated, systematic asymmetry in these traces, which may be due to (say) differences of the stiffness of the hindlimb during the Bottom trace shows the net angular displacements formed by 3 diodes as level in the sagittal (see inset), with minimum and maximum values corresponding to flexion and extension, resp. F) Peak to peak displacements in sagittal plane (L3 level) vs step cycle duration.

The straight lines for the Mf and VL onsets (“—— and —— respectively in B) as well as horizontal and sagittal displacements (in D and F) were constructed using the grouping method (... 1949) with 3 groups (slope = k).

The movements in the spine will be a consequence of the amount of internal as well as external forces acting on the trunk To estimate the intrinsic movements of the spine in the horizontal plane, the diode positions were compared with one another and if the angle between L5–L3–Th9 is plotted (see lowermost curve in Fig. 1 C), it turns out that lateral bending lies in the range of 10–15° However the movements of the ... (Fig. 1 C), i.e. the simultaneous movements of the three back markers, ar... The lateral movements decrease from about 40 mm ... 0 m

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EMG and movement data presented above suggest that the primary role of the muscles during walking is to control the stiffness of the back, rather than to induce ments.

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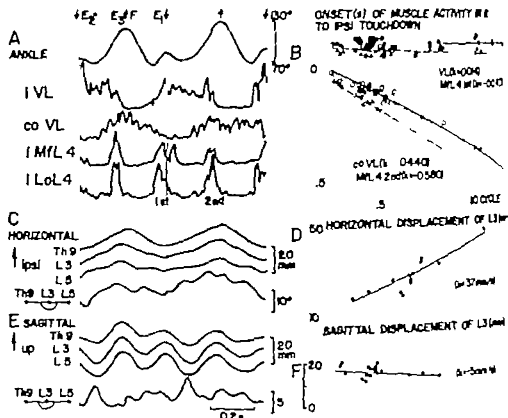


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Acta physiol. scand. 1979 105. 254–256

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Prostaglandin-mediated inhibition of noradrenaline release

VI On the intra-cardiac source of prostaglandins released from isolated rabbit hea

By

ÅKE WENNMALM

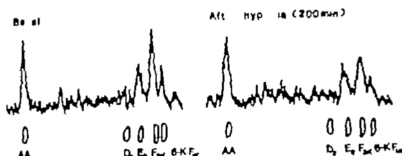
Prostaglandins (PG) of the D E, F and I series are released from isolated rabbit or g pig hearts, as shown in studies using thin layer chromatography (TLC, Isakson *et al* F gas chromatography (De Deckere *et al* 1977), or bioassay (Schrör *et al.* 1978) for ide cation of the PG released Although the physiological significance of cardiac PG is no established, various hypotheses have been presented Thus, PGI (prostacyclin) has suggested to play a role in coronary vasodilation in response to hypoxia (cf Needle and Kaley 1978), and PGE was recently proposed as a physiological modulator sympathetic transmitter release in the heart (Wennmalm 1978) Since these two differ endogenous PG are exerted to some extent on different intra-cardiac structures (coro vessels or sympathetic nerve terminals in the myocardium), we found it of interest to s whether PG formation in the heart is multifocal or alternatively is confined to one ty intra-cardiac tissue.

Rabbits of mixed strains and sexes, weighing from 2.0–3 0 kg, were used for the They were killed by a blow on the head and exsanguinated from the left carotid a The heart was rapidly removed and transferred to the perfusion apparatus, in wh was perfused according to Langendorff at a pressure of 60 cm H O with 37 C T solution The solution was aerated with 5% CO_2 in O_2. Some hearts were, in add prepared according to De Deckere *et al* (1977), which allows separate collection of co (Q_{cv}) and transmyocardial (Q_t) effluent.

In one series of hearts, ¹⁴C-arachidonic acid (¹⁴C AA New England Nuclear 40–60 mCi/mmole, 5 µCi), prepared as sodium salt was infused during 15 min cannula close to the aorta under continuous collection of effluent After the end infusion of isotope, perfusion was switched to a non-aerated, 37°C Tyrode so Perfusion with this anoxic solution was maintained during 75–200 min Followi anoxic period, perfusion was switched back to aerated Tyrode solution and a infusion of ¹⁴C-AA was performed as above, with continuous collection of pe

An another series the hearts after initial equilibration for 15–20 min in the pe apparatus, were infused with ¹⁴C-AA (10 µCi) during 30 min. Q_{cv} and Q_t were co continuously during the entire infusion.

The cardiac effluents were immediately acidified to pH 4.5 and extracted twic equal vol. of ether After washing of the pooled organic phases to neutral reacti



Radioscans (thin layer chromatography) of cardiac effluents collected during infusion of ^{14}C-arachi- *Left scan*: effluent collected under basal conditions from spontaneously beating heart. *Right scan*: at collected after 200 min perfusion with an un-aerated solution. The heart displayed no visible anical activity

oration to dryness, the residue was reconstituted in 0.2 ml ethanol and subjected to thin chromatography using 0.25 mm DC Kieselgel Fertigplatten F 254 (Merck) in solvent l acetate acetic acid 2,2,4-trimethyl-penthane water (90 20 50 100, v/v) against dards of AA, PGD_2, PGE_2, $PGF_{2\alpha}$ and 6-keto-PGF $_{1\alpha}$. Radioscans of the chromato- ms ere developed using a Berthold Dünnschichtscanner

fore anoxia, perfusion of the heart with ^{14}C-labelled sodium arachidonate yielded labelled PG in the effluent. The activity appeared chromatographically in two major s (apart from authentic ^{14}C-arachidonate), corresponding to $PGF_{2\alpha}$ and PGE_2, and smaller peak, corresponding to 6-keto-$PGF_{1\alpha}$ (Fig. 1). In most cases minor peaks also observed, drumning parallel with PGD and 13,14-dihydro-15-keto-PGE_2 (Me). ing the anoxic period the contractility of the myocardium rapidly faded, and after 20 min no visible mechanical activity of the organ occurred. Effluents collected during sion of ^{14}C-arachidonate after anoxia (maintained during 75, 90 and 200 min, respec ly) displayed mainly the same chromatographic pattern of ^{14}C PG as effluents obtained ore the anoxic period (Fig. 1), with respect to both the type and the amount of ^{14}C PG med

eparate collection of Q_{cv} and Q during infusion of ^{14}C-arachidonate yielded the owing data. Q constituted 1.9–2.4 % of the total cardiac effluent, and contained 1.0–4.0% the infused isotope that was not retained in the organ. In the Q effluents, 64 ± 14% ean S.E. n 3) of the activity was identified as ^{14}C PG in the Q_{cv} effluents the responding figure was 94 ± 3%. The relative proportions of the various ^{14}C-PG-peaks the Q_{cv} and the Q effluents did not differ in any respect from each other (Table I).

ble I Relative proportions of ^{14}C-PG in the coronary (Q_{cv}) and transmyocardial (Q_t) effluents from 3 hearts infused with ^{14}C-arachidonate. The data are presented as means ± S.E. of the ^{14}C-PG identified in the respective chromatograms. "Me" stands for 13,14-dihydro-15-keto-PGE_2.

	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	PGD_2	Me
	17 1	32 ± 3	26 ± 4	13 ± 3	11 ± 4
	19 2	32 5	29 ± 6	7 ± 1	13 ± 11

Comment

Sustained hypoxia (75–200 min) which completely annulled the contractility of the heart and probably also much of the remaining metabolic activity of the myocardial cells, did not have any effect on the profile of ^{14}C PG in the effluent during infusion of ^{14}C-arachidonic acid. This observation strongly suggests that the current effluent content of ^{14}C-PG was not the result of enzymatic transformation of ^{14}C AA in the myocardial cells. Excluding the heart muscle as a possible source of the cardiac PG formed from exogenous precursor draws attention to the alternative that the coronary vasculature was the origin of the PG synthesis. This concept is supported by the experiments in which slow passage of ^{14}C-arachidonic acid through the myocardium was not accompanied by a larger proportion of labelled metabolites in the effluent than that found after rapid passage via the coronary vasculature. The transmyocardial effluent (Q_t) did not differ in its ^{14}C PG profile from the coronary venous (Q_{cv}) effluent. In fact, the proportion of ^{14}C PG was smaller in the Q_t (64 %) compared to the Q_{cv} (94 %) effluent.

In summary, the present data strongly suggest that cardiac PG formation takes place mainly in the coronary vasculature. Since the adrenergic nerve terminals in the rabbit myocardium are distributed evenly throughout the tissue (Angelakos *et al.* 1963), it is evident that cardiac PG formation and sympathetic transmitter release are not strictly associated morphologically. This statement leads one to question the universal validity of the hypothesis (Hedqvist 1970, Wennmalm 1971) that sympathetic transmitter release is subject to a negative feed back control mediated by endogenously formed PG.

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Uptake and release of adenosine in isolated rat fat cells

By

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Abstract

DHOLM, B. B. and P HJEMDAHL. *Uptake and release of adenosine in isolated rat fat cells* Acta physiol. scand. 1979 *105* 257–267

…actively labelled adenosine and adenine were rapidly taken up by isolated rat fat cells, and incor…ated into nucleotides, of which ATP dominated. The overall process had an apparent K_m of 1 5 μM …ng incubation, especially in the presence of lipolytic agents, there was reduction in labelled ATP … compensatory increase in ADP AMP cAMP and nucleosides. The build-up of adenosine during …bation was inhibited by theophylline, which inhibits 5′-nucleotidase. Radioactivity released from …fused fat cells consisted mainly of nucleoside material, of which adenosine predominated. Lipolytic …ulation caused no significant increase in nucleoside outflow from perfused cells, whereas oxygenation … capable of reducing this outflow. It is concluded that adenosine is formed by fat cells as consequence …TP breakdown. Stimulation of lipolysis during activation of the sympathetic nerves leads to reversible …P breakdown and adenosine release. Adenosine might therefore act as modulator of lipolysis in vivo … these conditions, even though does not serve as feed back regulator in the proper sense.

…nosine has been ascribed a role as an endogenous modulator of cyclic AMP accumula… and lipolysis in adipose tissue (cf Fain 1973, Schwabe *et al.* 1975). Adenosine is formed …ng incubation of fat cells (Schwabe *et al.* 1973 1975), in perifused fat cells (Turpin *et al* …7) and in the *in situ* blood perfused canine subcutaneous adipose tissue (Fredholm 1976). …nosine is a potent inhibitor of cyclic AMP accumulation in fat cells (Fain, Pointer and …rd 1972, *Fain 1973 Ebert and Schwabe 1973 Hjemdahl and Fredholm 1976*). The …leoside also inhibits lipolysis in isolated rat fat cells provided that the concentration of lipolytic stimulus to be antagonized is kept low (Fain 1973 Fredholm 1978) and provided …t the level of endogenous adenosine is minimized either by using dilute cell suspensions …bert and Schwabe 1973) or by using a perfusion system (Turpin *et al.* 1977 Hjemdahl …d Sollevi 1978). Adenosine is also a potent inhibitor of lipolysis induced by sympathetic …ve stimulation *in situ* (Fredholm and Sollevi 1977).

Addition of adenosine deaminase, an enzyme that converts adenosine to inactive inosine, …ses lipolysis in isolated fat cells (Schwabe and Ebert 1974 Fain and Wieser 1975 Hjem…hl and Fredholm 1976). Moreover dipyridamol and dilazep, two inhibitors of adenosine …take, which lack antilipolytic effects by themselves potentiate the antilipolytic effect of

adenosine *in vitro* (Fredholm 1978) and inhibit lipolysis induced by nerve stimulation [illegible] (Fredholm and Sollevi 1977 1978) These findings lend further support to the [illegible] endogenous adenosine might act as a modulator of lipolysis.

It was originally suggested that adenosine might act as a feedback regulator of lip[illegible] (Fain *et al* 1972) In order to demonstrate such a feedback inhibitory role release of [illegible] sine by lipolytic stimuli must be demonstrated Schwabe and coworkers (1973) demonstr[illegible] accumulation of adenosine in the fat cell incubates but were unable to show a signific[illegible] enhanced accumulation in the presence of lipolytic hormones. Fain and Shepherd [illegible] have been similarly unsuccesful in demonstrating a consistent effect of lipolytic drug[illegible] adenosine release. Furthermore, in the intact perfused canine subcutaneous adipose [illegible] sympathetic nerve stimulation induced release of adenosine-like material, which wa[illegible] hibited by α-adrenoceptor antagonists which actually potentiate the lipolytic re[illegible] (Fredholm 1976)

The aim of the present studies has been to further examine the question if lipolytic [illegible] mones release adenosine, and consequently if this nucleoside may be considered as [illegible] feed back regulator of lipolysis. Some of these findings were communicated in prelim[illegible] form at the meeting of the Scandinavian Physiological Societies in November 1977 (Hje[illegible] dahl *et al* 1978)

Materials and methods

Epididymal fat pads from 2 5 decapitated Sprague Dawley rats (Anticimex strain) weighing 1[illegible] were pooled in each experiment Isolated fat cells were prepared as described by Rodbell (1964), by [illegible] tion with 3 mg/ml crude bacterial collagenase (Worthington) for 40–60 min. The cells [illegible] times before prelabelling or incubation All incubations were carried out in Krebs-Ringer phosphate [illegible] pH 7 4 cont [illegible]ining 5.5 mM glucose 1.8 mM $CaCl_2$ and 3 per cent bovine serum album[illegible] (fraction [illegible] St Louis)

Cyclic AMP was determined in the fat cell incubates with the protein binding assay described by [illegible] Eakins and Albano (1972) afte[illegible] treatment of the samples as described by Fredholm and Hjemdahl [illegible] Thus cells and medium were precipitated by perchloric acid (0.4 N final concentration). Following [illegible] fugation the supernatant was neutralized by 4 N KOH and Tris base was added to a f[illegible]nal concentration [illegible] 50 mM The potassium perchlorate precipitat[illegible] was removed by centrifugation after [illegible]hich cyclic [illegible] was determined on the supernat[illegible]t

Prelabelli[illegible]g of fat cells was performed by incubating a concentrated cell suspension for [illegible] 10–100 μCi [2-³H]-adenine (New England Nucle[illegible] 23 0 Ci/mmol) or [8 ¹⁴C]-adenine (43.95 mCi/[illegible] after which the cells were washed 3–6 t[illegible]mes before subsequent incubations. In uptake experiments [illegible] adenine [illegible]C-adenine or [2,8 [illegible]H]-adenosine (New England Nucle[illegible] 30.4 Ci/mmol) [illegible]ere added to [illegible] cell suspension at time 0. Aliquots of this suspension were subsequently removed at the indicated [illegible] treated as described [illegible]bove for the determination of cyclic AMP

The radioactive purines were separated by ion-excha[illegible]ge on thin layer chromatography Group [illegible] tion into nucleotide [illegible]nd non-nucleotide material was effected by chromatography on small (2.5 [illegible] columns of Dowe[illegible] 1 [illegible] 2 (200–400 mesh) esse[illegible]tially as described by Rutte[illegible] *et al.* (1973). Separation [illegible] different nucleotides wa[illegible] performed [illegible]ither by chromatography on long anion-exchange columns, a[illegible] scribed previously (Fredholm *et al* 1977) or by chromatography on PEI TLC sheets as described by [illegible] and Schultz (1974). Nucleosides and bases w[illegible]re separated by thin-layer chromatography on silica [illegible] described by Pull and McIlwain (1972).

Perf[illegible]sion experiments. Fat cells were isolated and prelabelled with [illegible]H-adenine as described [illegible] After the final washing 1 ml of packed cells [illegible]a[illegible] transferred to a water-jacketed plastic chamber [illegible] 2 2 5 ml Krebs-Ri[illegible]ger phosphate buff[illegible]r pH 7 4 containing 1.8 mM $CaCl_2$ 5.5 mM glucose and [illegible] serum albumin. The floating f[illegible]t cells were subsequently "perfused" (Allen *et al* 1973) with this buffer [illegible] 37°C and a rate of 2 ml/min Noradrenaline [illegible]r isoprenaline were diluted in saline containing 20 μg [illegible]

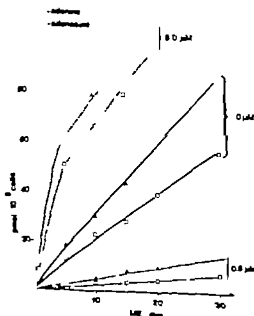
...ic acid and infused via ... side arm before the perfusion chamber. With the present technique ... steady ... concentration of drug is obtained ... within 5 min of infusion and lipolysis, measured as glycerol produc... approaches steady state levels after 15–20 min of stimulation (Hjemdahl 1976). ³H-radioactivity in ... and perfusion effluent was characterized and measured as described above.

...nucleotidase (EC 3.1.3.5) was determined according to Gentry and Olsson (1975) as described by ...olm *et al.* (1978). Fat cells from tw... rats ...ere homogenized in 5 ml 60 mM Tris-acetate pH 7.4. ... centrifugation at 1 600 ... g for 10 min the fat free supernatant was removed and diluted 5 times ...ith ... same buffer. 0.05 ml of the extract ...as incubated for 15 min at 37°C ...ith ¹⁴C 5'-adenosine mono... ...phate (1–2 500 μM final concentration) in ... total volume of 1 ml 60 mM Tris-acetat... pH 7.4 containing ... M magnesium acetate. The assays were performed either in the presence or the absence of 25 mM ...ethylene adenosine diphosphate (AOPCP, PL-biochemicals). The reaction ...as stopped by the addi... ...of 0.2 ml 0.15 M Zn SO_4 and 0.2 ml 0.15 M Ba $(OH)_2$. Following centrifugation (2 000 ... g for 10 min) ...activity in 0.9 ml supernatant was determined.

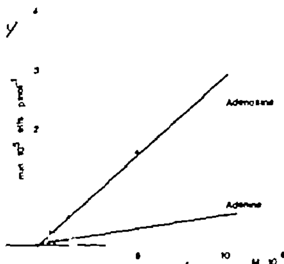
Results

...nine and adenosine were rapidly incorporated into nucleotides in the fat cells. The ... course and concentration dependence is shown in Fig. 1. At low concentrations the ... of incorporation was essentially linear for 30 min, while at higher concentrations (...r 2 μM) there was a rapid initial rate of incorporation followed by a slower rate. The ...ial rate of incorporation was calculated in several experiments and their concentration ...endence was plotted according to Lineweaver and Burk. Results from one typical experi...t is shown in Fig. 2. The apparent K_m ranged between 1 and 5 μM for both substrates. ... apparent V_{max} was 30–60 pmol/min/10⁶ cells for adenine and 5–6 times less for adenosine (...15 pmol/min/10⁶ cells).

...he radioactivity taken up by fat cells after labelling with adenine and before incubation ... mainly in the form of nucleotides—of which ATP dominated (Fig. 3). During incuba-



...g. 1. Time-course of incorporation of la...lled adenine and adenosine into nucleotides ... fat cells. Results are duplicate determina...tions from ... single expt.



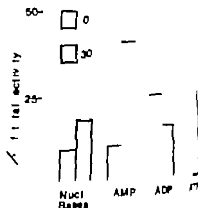


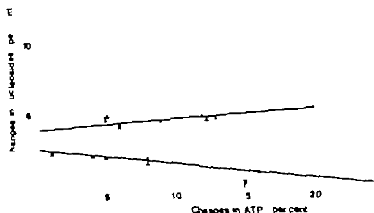
Fig. 2. Double reciprocal (Lineweaver Burk) plot of the initial rate of adenine and adenosine incorporation into nucleotides.

Fig. 3 Distribution of radioactivity in fat cells before and after incubation for 30 min. Fat cells were prepared from 5 rats, incubated for 10 min with 10 μCi ^{14}C-adenine washed 6 times and incubated for min in Krebs-Ringer phosphate buffer containing 0.1 mM dipyridamol. Radioactivity in the different fractions was determined by chromatography on long (0.8 15 cm) Dowex 1 2 (200–400 mesh) columns. The cells were incubated either in control buffer or in buffer containing 10 μM noradrenaline. There were no significant differences

tion there was a progressive fall in ATP and a compensatory increase in mainly AMP in the nucleoside fraction When the cells were incubated in the presence of dipyridamol (0 1 mM) there was a pronounced change in the distribution already after 30 min (Fig. In the absence of dipyridamol the changes were qualitatively similar but less pronounced (not shown)

The formation of nucleosides (adenosine and inosine) is dependent upon dephosphorylation of AMP The hydrolysis of AMP in fat cell homogenates was inhibited by approximately 70 per cent by AOPCP suggesting that 5 nucleotidase is the major enzyme responsible for the hydrolysis in these cells. The apparent K_m for the reaction was 35 μM and the apparent V_{max} was 75 nmol min g essentially in agreement with the results of Newby et al (1975) The rate of formation of nucleosides and bases is likely to increase with decreasing ATP levels for two reasons. Firstly ATP is a potent inhibitor of the enzyme (cf N *et al* 1975) Secondly the amount of substrate, AMP is increased when ATP hydrolysis is increased As shown in Fig. 4 such a relationship could be detected, even though it was of borderline significance, provided that there was no theophylline present in the incubation. The apparent inhibition of purine formation by theophylline may have at least two explanations.

In agreement with data obtained in brain and kidney (Tzusuki and Newburgh Fredholm Hedqvist and Vernet 1978) we found that 1 mM theophylline inhibits



4 The relationship between changes in ^{3}H-ATP and in ^{3}H-purines during 30 min incubation of fat The distribution of radioactivity after labelling with ^{3}H-adenine was determined. At the beginning incubation 48 ± 1 of the radioactivity was in the form of ATP and 1 ± 1% in the form of nucleosides [illegible]. The cells were then incubated with or without different drugs in the presence of no phosphodiesterase inhibitor (Δ), in the presence of 0.1 mM papaverine (●) or in the presence of 1.0 mM theophylline The relationship was calculated with conventional linear regression analysis. In the presence of no phosphodiesterase inhibitor or papaverine there was positive correlation ($r = 0.358$, $p < 0.05$), in the [presence] of theophylline there was significant negative correlation ($r = -0.679$, $p < 0.005$). The linear [regression] lines of y on x are indicated

[...]esterase by 65 per cent in fat cells. Secondly theophylline may by diverting ATP [meta]bolism to cyclic AMP channel labelled purines away from nucleotide formation. The [result]s presented in Table I demonstrate the pronounced elevation of cyclic AMP levels [whe]n theophylline, but not papaverine, was combined with β-adrenoceptor stimulating [dru]gs.

[T]he results in Table II demonstrate that noradrenaline and isoprenaline decreased H-ATP [in fa]t cells labelled with ^{3}H-adenine. The effect of 3 µM noradrenaline was diminished, but [not e]liminated by 4 µM propranolol. This dose of propranolol similarly was not sufficient

[Table] I Effect of noradrenaline (NA), isoprenaline (Iso) and methoxamine on adipocyte cyclic AMP levels. The cells were incubated for 5 min in the presence of theophylline (1.0 mM) or papaverine (0.1 mM) followed by 15 min in the presence of the drugs indicated. Mean ± S.E. Number of observations in parentheses.

	Cyclic AMP content pmol/10⁶ cells	
	Theophylline	Papaverine
[No] drug	3.7 ± 0.3 (8)	3.4 ± 0.6 (8)
[NA] (3 µM)	128.1 ± 14.7 (4)	10.9 ± 1.2 (4)
[+] propranolol (4 µM)	12.4 ± 1.7 (4)	3.2 ± 0.7 (4)
[Iso] (0.3 µM)	134.1 ± 15.5 (4)	10.2 ± 0.4 (4)
[+] propranolol (4 µM)	67.5 ± 11.1 (4)	3.2 ± 0.9 (4)
[Me]thoxamine (70 µM)	5.4 ± 0.8 (4)	2.1 ± 0.3 (4)

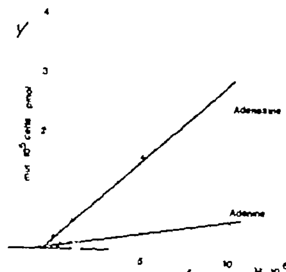


Fig. 2

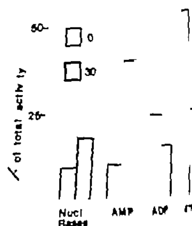


Fig. 3

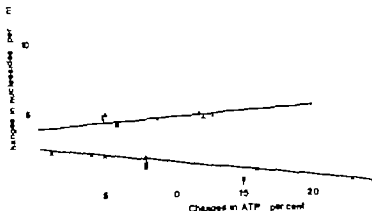
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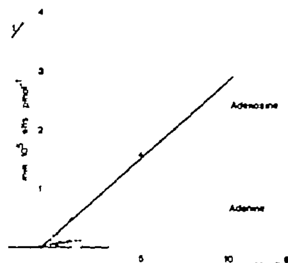
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... (0.3 μM)	134.1±15.5 (4)	10.2±0.4 (4)
... propranolol (4 μM)	67.3±11.1 (4)	3.2±0.9 (4)
...thoxamine (70 μM)	5.4±0.8 (4)	2.1±0.3 (4)



F g. 2

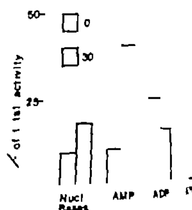


Fig. 3

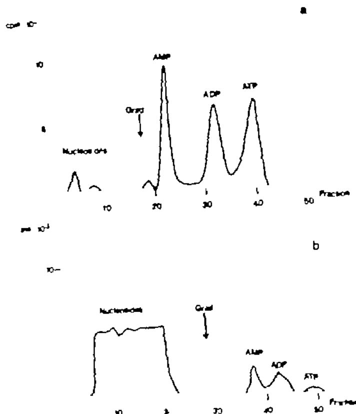
Fig. 2. Double reciprocal (Lineweaver Burk) plot of the initial rate of adenine and adenosine incorp into nucleotides.

Fig. 3 Distribution of radioactivity in fat cells before and after incubation for 30 min. Fat cells wer pared from 5 rats, incubated f 10 min with 10 μCi ^{14}C-adenine washed 6 times and incubated b min in Krebs-Ringer phosph te buffer containing 0.1 mM dipyridamol. Radioactivity in the di fraction was determined by chromatography on long (0.8 15 cm) Dowex 1 2 (200–400 mesh) col The cells were incubated either in control buffer or in buffer containing 10 μM noradrenaline. Ther no significant diff rences.

tion there was a progressive fall in ATP and a compensatory increase in mainly AMP in the nucleoside fraction When the cells were incubated in the presence of dipyrid (0.1 mM) there was a pronounced change in the distribution already after 30 min (Fig In the absence of dipyridamol the changes were qualitatively similar but less pronoun (not shown)

The formation of nucleosides (adenosine and inosine) is dependent upon dephospho tion of AMP The hydrolysis of AMP in fat cell homogenates was inhibited by approxim 70 per cent by AOPCP suggesting that 5 nucleotidase is the major enzyme responsib the hydrolysis in these cells. The apparent K_m for the reaction was 35 μM and the app V_{max} was 75 nmol min g essentially in agreement with the results of Newby (1975) The rate of formation of nucleosides and bases is likely to increase with decre ATP levels for two reasons. Firstly ATP is a potent inhibitor of the enzyme (*cf* N *et al* 1975). Secondly the amount of substrate, AMP is increased when ATP hydroly increased. As shown in Fig. 4 such a relationship could be detected even though it w borderline significance, provided that there was no theophylline present in the incubat The apparent inhibition of purine formation by theophylline may have at least two exp tions.

In agreement with data obtained in brain and kidney (Tsuzuki and Newburgh Fredholm Hedqvist and Vernet 1978) we found that 1 mM theophylline inhibits



6 A. Distribution of radioactivity in fat cells prelabelled with ^{3}H-adenosine. The separation was performed on long Dowex 1 column eluted with hydrochloric acid gradient.
6 B. Distribution of radioactivity in the perifusion effluent from the same cells as in Fig. 6 A. Note marked shift towards nucleosides in the effluent, as compared to the distribution in the cells.

7) and noradrenaline had no significant further effect (to 11.8 ± 2.6 %, n = 5). When the ... were incubated with theophylline the nucleoside release was smaller in magnitude (see ... 4) and there were no consistent effects of catecholamines.

The fall in ATP commonly seen following incubation with lipolytic drugs has been attributed to accumulation of lipolytic products, primarily fatty acids (Angel *et al* 1971). In order to study a possible direct effect of noradrenaline on purine release from fat cells we therefore used the perifused fat cell system (Allen *et al* 1973, Hjemdahl and Sollevi 1978). This system has the advantage that lipolytic products do not accumulate since the medium is constantly changed.

After the initial equilibration period of 10-15 min there was a fairly steady outflow of radioactivity from ^{3}H-adenine prelabelled perifused fat cells (Fig. 5). This basal outflow of radioactivity amounted to 0.49 ± 0.04 % of the cellular content per min (n = 11). As can be seen in Fig. 5 most of the radioactivity released was nucleoside material, of which adenosine

TABLE II Distribution of radioactivity as ATP and as nucleosides and bases after incubation with ... Incubation for 45 min in the presence of papaverine + indicated drug. Mean of 4-6 ... tions. Results are expressed as per cent of total radioactivity

	ATP	Nucleosides & Bases
Control	40.5 ± 0.6	3.9 ± 0.6
NA 3 μM	27.5 ± 0.5*	6.3 ± 0.3
NA + propranolol 4 μM	34.0 ± 2.0	4.6 ± 0.1
Iso 0.3 μM	33.0 ± 6.0	5.8 ± 1.0
Iso + propranolol 4 μM	31.5 ± 1.5	4.6 ± 0.3
Methoxamine 70 μM	38.0 ± 1.0	5.8 ± 0.6

$p < 0.01$ vs. control
p 0.01 vs. no propranolol

to completely block the action of noradrenaline on cyclic AMP (Table I). Noradren... also increased the fraction of radioactivity recovered in the form of nucleosides and ... significantly when the cells were incubated with papaverine (Table II). This effect of ... adrenaline was antagonized by propranolol. When the cells were incubated in the pres... of dipyridamol (0.1 mM) the accumulation of purine nucleosides was higher (10.2 ...

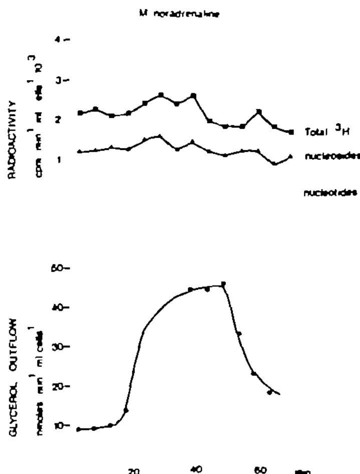


Fig. 5. Lipolysis and outflow of radioactivity from ^{3}H-adenine labelled perirenal fat cells stimulated by 1 μM noradrenaline. Radioactivity was separated into nucleoside and nucleotide fractions ... small Dowex 1 ... umns. Glycerol ... as ... described by Hjemdahl ... Note the very minor change in nucleoside outflow during stimulation with ... dose of noradrenaline causing ... substantial lipolytic response

·) argument that it acts—at least partly—by interfering with endogenous adenosine *et al* 1972, Ebert and Schwabe 1973).

..belled adenosine accumulated in the incubation medium, and under some circumstances ..ccumulation was enhanced by noradrenaline. Moreover the effect of noradrenaline antagonized by propranolol. However the purine formation may not necessarily by ..ed to lipolysis. During these conditions there was a marked reduction of fat cell ATP ·, in agreement with the results of others (Hepp *et al.* 1968 Bihler and Jeanrenaud .., Angel *et al.* 1971) It has been shown that such a depression of ATP levels may be ..ed by lipolysis in so far as it appears to be caused by accumulation of fatty acids (Angel .. 1971). The present finding that there was very little increase in purine release by ..drenaline in perifused fat cells where accumulating fatty acids are continuously washed ..y may be taken as an argument that the noradrenaline-induced nucleoside accumula- .. in incubated fat cells is not directly caused by lipolysis. Furthermore, when the cells .. incubated in the presence of dipyridamol or theophylline no consistent effects of lytic catecholamines on purine nucleoside accumulation were found. Similarly Schwabe *l.* (1973) were unable to detect a significant effect of noradrenaline on adenosine levels .. cell incubates.

..hen lipolytic stimuli increase accumulation of purine nucleosides, including adenosine, may thus be due to the depression of ATP levels that can be caused by accumulating ..ytic products such as fatty acids, rather than to a direct effect of the lipolytic stimuli. cell ATP levels may also be depressed by decreased oxygen supply The finding that ..genation, in contrast to mere aeration, of the perifusion buffers led to a decreased rate ..of the nucleoside release suggests that the oxygen supply is also of importance.

the intact adipose tissue adenosine-like material was released by sympathetic nerve ..ulation (Fredholm 1976). This release was prevented by α-adrenoceptor blockade. The ..tionship between decrease in ATP levels and enhanced purine nucleoside release has .. demonstrated also under *in vivo* conditions (Fredholm *et al.* 1977). It is interesting to .. that during sympathetic nerve stimulation, which causes vasoconstriction concomitantly .. lipolysis, there is a decrease in tissue pO (Fredholm *et al.* 1976) as well as an increase .. the tissue content of FFA (Fredholm 1970). Sympathetic nerves provide the most im- ..rtant stimulus for lipolysis *in vivo* Therefore, ATP levels may decrease during activation of ..olysis which, in turn, will lead to increased formation of adenosine.

The present findings thus support the idea that adenosine is not a feed back regulator of ..olysis in the strict sense of the word. The formation of adenosine is not causally linked ..lipolysis, but does appear to be linked to changes in cellular ATP levels. However re- ..sible changes in ATP are caused by the same stimuli that cause lipolysis. A role for ..enosine as a physiological modulator of lipolysis thus remains a distinct possibility

..is study .. supported by the Swedish Medical Research Council (04X 2553). Magnus Bergvalls stiftelse ..d by Karolinska Institutet. W.. want to thank Miss Ann-Sofie Larsson and Mrs Louise Vernet for expert ..chnical assistance and Mrs Birgitta Pihap for assistance in preparing the manuscript. Dipyridamol was ..gift from Boehringer Ingelheim.

was a major component (thin layer chromatography not shown). Fig 6 a shows the dis- tion of the radioactivity in an aliquot of prelabelled cells and Fig. 6 b the distribution radioactivity in the perifusion effluent from the same cells. It can be seen that most of radioactivity in the cells is present in the nucleotide stores, whereas most of the [illegible] activity in the effluent is nucleoside material.

Stimulation of lipolysis in the perifused fat cells by 1 μM noradrenaline caused [illegible] minor if any increase in nucleoside outflow as shown in Fig. 5 On the average 1 μM adrenaline increased outflow of radioactivity from ³H-adenine prelabelled fat cells by [illegible] 12 ± 6 (n = 8 N.S.). Similarly 1 μM isoprenaline had an insignificant effect on out- (increase by 14 ± 13 %, n = 4) The outflow of nucleosides was, however influenced by [illegible] oxygenation of the perifusion buffer Thus, the fractional release of radioactivity [illegible] reduced by 34 ± 7 (n = 5 p < 0 01) in cells perifused with oxygenated buffer as com- to identical cells perifused with aerated buffer The lipolytic response of the cells was [illegible] consistently altered by this change in PO_2.

Discussion

The present study confirms and extends earlier reports of rapid uptake and phosphor[illegible] of adenine and adenosine (Kuo and De Renzo 1969 Ebert and Schwabe 1973). The [illegible] incorporation into nucleotides was faster with adenine as substrate and consequently [illegible] was used for subsequent studies of the formation of purines from prelabelled nucle[illegible] stores.

Already during basal conditions (*i.e* in the absence of lipolytic drugs) there was [illegible] preciable formation of purine nucleosides. In the perifused fat cell system, where [illegible] nucleosides are continuously removed, the rate of formation amounted to about 0.5 [illegible] the total cellular radioactive stores per min Assuming homogenous labelling of the [illegible] tide stores and an ATP content of 1–2 nmol/10⁶ cells (Schwabe *et al* 1974, Fredholm [illegible] 1977) this would correspond to the release of 5–10 pmol adenosine/min/10⁶ cells. [illegible] estingly this figure agrees well with the initial rate of accumulation of endogenous [illegible] sine during the incubation of fat cells (Schwabe *et al* 1973)

The accumulation of purines was markedly inhibited by theophylline. Thus, the [illegible] interferes with adenosine mechanisms in fat cells in several different ways. (1). The lipolytic and cyclic AMP depressing effect of adenosine is competetively inhibited by [illegible] phylline (Fain 1973 Ebert and Schwabe 1973 Fredholm 1978), (2) the formation of [illegible] sine is inhibited by theophylline at least partly due to inhibition of 5'-nucleotidase (T[illegible] and Newburgh 1975 Fredholm *et al* 1978), and (3) theophylline may inhibit ade[illegible] uptake by fat cells (Fain *et al* 1978). Although the latter effect is minor (Schwabe [illegible] 1973), it may reflect hindrance not only of inward but also outward transport of p[illegible] over the fat cell membrane. These findings further underscore the hazards of assum[illegible] theophylline acts exclusively by virtue of its (poor) phosphodiesterase inhibitory [illegible] The finding that theophylline is far more potent as a stimulator of cyclic AMP acc[illegible] tion in fat cells than the considerably more potent phosphodiesterase inhibitors pap[illegible] and dipyridamol (Schwabe *et al* 1972, Hjemdahl and Fredholm 1976) may instead [illegible]

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F ords, the Spitzbergen reindeer is much fatter i the fall than their Norwegian relatives. ..eads to the question of whether such marked fattening capability is reflected in a ¹⁰onal pattern different from that of other reindeer

n, the purpose of the present study was to determine the levels of growth hormone yroxine in free ranging Spitzbergen reindeer both during late winter when food is in supply and during late summer before the rut, when their nutritional status is optimal. dition, the concentration of cortisol was to be measured as a tentative indicator of

Material and methods

of 33 Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) ere used. Blood samples were collected two expeditions to Svalbard in 1976. During the first expedition (Adventdalen, March 24 April 8, ate-winter blood samples were collected from 30 adult animals (20 females and 10 males) and from s accompanying their mothers. The samples were taken by jugular venopuncture using evacuated ng tubes, immediately after capture of the animals. During the second expedition (Grondjorden, 13–27 1976) late-summer blood samples ere collected as described above from 14 adul animals ales and 5 males) and from 5 calves accompanying their mothers. The serum fractions were frozen ored at 18°C till analysis.

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roxine (T_4) as measured according to the method of Webb (1972) as modified by Foss (1973) tisol in undiluted serum samples was determined by Cortipack® Assay Kits (Amersham, England). rences between mean values were tested statistically by use of Student t-test. results are presented as Mean ± S E.

Results

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Fig. 1 the summer–winter variation in growth hormone for the Spitzbergen and Nor an reindeer is shown. A conspicuous (P 0.01) increase in the level of growth hormone dent in winter both in calves and in adult Spitzbergen reindeer 10.8 ± 1 4 ng/ml 5) vs 71.5 19 ng/ml (n 4) for calves, and 11.2 ± 0.5 g/ml (n – 14) and 73.3 ± 11 ng/ml 30) for adults. Winter levels of growth hormone were higher in Spitzbergen reindeer in Norwegian reindeer 21.6 ± 1.6 ng/ml (calves, n – 14) and 20.3 ± 0.9 ng/ml (adults, 9) in the latter while summer levels were higher in the Norwegian reindeer 31.9 ± 2.2 'd for calves (12) and 23.3 ± 1.3 ng/ml for adults (n 24). Seasonal changes in growth none in Norwegian reindeer were small, but summer levels tended (P 0.05) to be ewhat higher than winter levels.

o seasonal change in cortisol levels were recorded for the adult groups. 5 7 ± 0.6 µg/100 n 14) and 4 7 0.5 µg/100 ml (21) for Spitzbergen and Norwegian reindeer re tively in summer and 5.8 _0.3 µg/100 (n 30) and 5 1 ± 0 8 µg/100 ml (n 15) for the responding groups in winter The Norwegian calf group was generally lower in cortisol t the other groups. 2.8 0 3 µg/100 ml (n 1.) (P 0 01) during summer and 3.5 ± 0.5 100 ml (n 13) (P 0 05) during winter while the Spitzbergen calf group did not differ nificantly from the adult groups 5.0 ± 1 7 µg/100 ml (n 5) (summer), and 6.7 ± 0.7 µg/ ml (n 4) (winter) (Fig. 2).

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The Spitzbergen reindeer—a winter-dormant ungulate?

By

TATA RINGBERG

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Abstract

RINGBERG T *The Spitzbergen reindeer—a winter-dormant ungulate?* Acta physiol sc. 1979 *105* 268–273

Seasonal changes in serum levels of growth hormone cortisol and thyroxine in calves and adult Spitzber reindeer (*Rangifer tarandus platyrhynchus*) were measured and compared to those previously f Norwegian reindeer (*R.t. tarandus*). Cortisol did not differ significantly between summer and win between the subspecies. Growth hormone and thyroxine exhibited highly significant seasonal changes subspecific differences winter levels of growth hormone were much higher than summer levels Spitzbergen reindee while Norwegia eindeer differed very little from season to season. Both S bergen and Norwegian reindeer had markedly reduced thyroxine levels during winter but the values the former were much lower than those from the latter In summer however the levels are equal. T high lev ls of growth hormone and low levels of thyroxine in the Spitzbergen reindeer during win indicative of high lipolytic activity nd reduced metabolic rate respectively

The Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) occupy one of the northern habitats of all cervidae, and have been isolated on the archipelago of Svalbard (Spitzber for an unknown number of centuries. The climate of Svalbard is influenced by a nort going branch of the Gulf Stream and the temperatures therefore do not reach the extrem of more typical high-arctic areas Strong winds of high moisture and occasional peri of above-freezing temperatures in late winter however result in icing of the snow and covering the range and reduce the amount of vegetation accessible to the reindeer (Reim 1977). Also the search for food may be impaired by a winter night of almost compl darkness for nearly 3 months. Consequently the food availability is limited and the animal must heavily rely upon the fat reserves gained and stored during summer for their win survival.

Studies on the body composition of the Spitzbergen reindeer in late summer (Reim *et al* 1977) have shown the fat/muscle ratio to be much higher than that of Norweg reindeer (Ringberg, unpublished) although the total body weight is about the same I

Mean temp. for the warmest month, July is +6°C, and for the coldest month, December is –1 Data from the Stat Meteorological Institut of Norway

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Abstract

RINGBERG T *The Spitzbergen reindeer—a winter-dormant ungulate?* Acta physiol sc 1979 *105* 268-273

Seasonal changes in serum levels of growth hormone cortisol and thyroxine in calves and adult Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) were measured and compared to those previously found in Norwegian reindee (*R.t tarandus*). Cortisol did not differ significantly between summer and winter nor between the subspecies. Growth hormone and thyroxine exhibited highly significant seasonal changes and subspecific differences. Winter levels of growth hormone were much higher than summer levels in Spitzbergen reindeer while Norwegian reindeer differed very little from season to season. Both Spitzbergen and Norwegian reindeer had markedly reduced thyroxine levels during winter but the values of the former were much lower than those from the latter In summer however the levels were equal. The high levels of growth hormone and low levels of thyroxine in the Spitzbergen reindeer during winter are indicative of high lipolytic activity and a reduced metabolic rate respectively

The Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) occupy one of the northernmost habitats of all cervidae, and have been isolated on the archipelago of Svalbard (Spitzbergen) for an unknown number of centuries. The climate of Svalbard is influenced by a north-going branch of the Gulf Stream and the temperatures therefore do not reach the extremes of more typical high-arctic areas Strong winds of high moisture and occasional periods of above freezing temperatures in late winter however result in icing of the snow surface covering the range and reduce the amount of vegetation accessible to the reindeer (Reimers 1977). Also the search for food may be impaired by a winter-night of almost complete darkness for nearly 3 months. Consequently the food availability is limited and the animals must heavily rely upon the fat reserves gained and stored during summer for their winter survival

Studies on the body composition of the Spitzbergen reindeer in late summer (Reimers *et al* 1977) have shown the fat/muscle ratio to be much higher than that of Norwegian reindeer (Ringberg, unpublished) although the total body weight is about the same [illegible]

Mean temp. for the warmest month July is +6 C, and for the coldest month, December, is [illegible] Data from the State Meteorological Institute of Norway

words, the Spitzbergen reindeer is much fatter in the fall than their Norwegian relatives. ...ads to the question of whether such marked fattening capability is reflected in a ...al pattern different from that of other reindeer

...the purpose of the present study was to determine the levels of growth hormone ...rmone in free ranging Spitzbergen reindeer both during late winter when food is in ...upply and during late summer before the rut, when their nutritional status is optimal. ...tion, the concentration of cortisol was to be measured as a tentative indicator of ...

Material and methods

...of 53 Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) were used. Blood samples were collected ...two expeditions to Svalbard in 1976. During the first expedition (Adventdalen, March 24 April 8, ...te-winter blood samples were collected from 30 adult animals (20 females and 10 males) and from ...accompanying their mothers. The samples ere taken by jugular venopuncture using evacuated ...ng tubes, immediately after capture of the animals. During the second expedition (Groesfjorden, ...13–27 1976) late-summer blood samples ere collected as described above from 14 adult animals ...les and 5 males) and from 5 calves accompanying their mothers. The serum fractions ere frozen ...red at 18°C till analysis.

...th hormone as measured by the radioimmunoassay of Wool and Selenkow (1968) as modified ...s and Blom (1973). Bovine growth hormone (a gift from NIH-Bethesda, Batch no. B1003A) was ...standard, and rabbit anti-BGH as anti-serum.

...xine (T_4) was measured according to the method of Webb (1972) as modified by Foss (1973).

...sol in undiluted serum samples was determined by Cortipack Assay Kits (Amersham, England).

...rences between mean values were tested statistically by use of Student's t-test.

...sults are presented as Mean ± S.E.

Results

...ifference between sexes was found for any of the hormones tested. The results from ...s and females are therefore pooled.

Fig. 1 the summer–winter variation in growth hormone for the Spitzbergen and Nor...an reindeer is shown. A conspicuous (P 0.01) increase in the level of growth hormone ...ident in winter both in calves and in adult Spitzbergen reindeer 10.8 ± 1.4 ng/ml ...5) vs 71.5 19 ng/ml (n 4) for calves, and 11.2 ± 0.5 ng/ml (n 14) and 73.3 ± 11 ng/ml ...30) for adults. Winter levels of growth hormone were higher in Spitzbergen reindeer ...n Norwegian reindeer: 21.6 1.6 ng/ml (calves, n 14) and 20.3 ± 0.9 ng/ml (adults, ...9) in the latter while summer levels were higher in the Norwegian reindeer: 31.9 ± 2.2 ...for calves (12) and 25.5 ± 1.5 ng/ml for adults (n 24). Seasonal changes in growth ...mone in Norwegian reindeer were small, but summer levels tended (P 0.05) to be ...what higher than winter levels.

...seasonal change in cortisol levels were recorded for the adult groups 5.7 ± 0.6 µg/100 ...n 14) and 4.7 0.5 µg/100 ml (n 21) for Spitzbergen and Norwegian reindeer re...tively in summer and 5.8 0.3 µg/100 (n 30) and 5.1 _0.8 µg/100 ml (n = 15) for the ...rresponding groups in winter. The Norwegian calf group was generally lower in cortisol ...the other groups 2.8 0.3 µg/100 ml (n 12) (P 0.01) during summer and 3.5 ± 0.5 ...100 ml (n 13) (P 0.05) during winter while the Spitzbergen calf group did not differ ...ificantly from the adult groups, 5.0 1.7 µg/100 ml (n 5) (summer), and 6.7 ± 0.7 µg/ ...ml (n 4) (winter) (Fig. 2).

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The Spitzbergen reindeer—a winter-dormant ungulate?

By

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Abstract

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Mean temp. for the warmest month, July is +6°C, and for the coldest month, December, is Data from the State Meteorological Institute of Norway

…g. 3 it is shown that the thyroxine level in the two calf groups are identical during …r 261 ± 17 nmol/l (n 5) for Spitzbergen, and 249 ± 10 nmol/l (n = 12) for Norwegian … and likewise, that the adult level of thyroxine during summer is similar: 216 ± 15 … (n 14) for Spitzbergen and 223 ± 7 nmol/l (= 24) for Norwegian reindeer. In … the thyroxine levels of both subspecies are markedly reduced, but the decrease in …itzbergen reindeer is considerably larger than that in Norwegian reindeer: 69 ± 7 …l (n 4) vs. 144 ± 9 nmol/l (n 13) for Spitzbergen and Norwegian calves, respectively … winter and 80 _5 nmol/l (n 30) vs. 157 ± 5 nmol/l (n = 16) for adults, respectively … the winter thyroxine concentration in the Spitzbergen reindeer is only about one … of the summer concentration and 50% lower than the winter levels of Norwegian …er. The seasonal changes are statistically highly significant P < 0.001 for Spitzbergen …er and P 0.01 for Norwegian reindeer.

Discussion

…th hormone plays a key role in maintaining blood homeostasis of fatty acids and …e by stimulating the mobilization of free fatty acids from triglycerides in depot fat … by stimulating the mobilization of glucose from the liver and by exerting a marked …sulin effect (Glick *et al.* 1965 Talwar *et al.* 1975). The high growth hormone con…ations in winter blood samples from Spitzbergen reindeer are therefore strongly sug…e of an energy metabolism which is dependent upon the mobilization of stored fat to …ch higher degree than that for Norwegian reindeer.

…wever during periods of stress such as pain, starvation or exposure to cold, the level …owth hormone is often elevated as part of the physiological response to stress (Roth … 1963 Feldman and Brown 1967 Olbich and Trenkle 1973). Thus, the high growth …one levels could also indicate, that the winter conditions on Svalbard with scarce … darkness and high windchill are tough on the animals, rather than being reflections …justments of energy metabolism.

…hen an animal is exposed to a variety of noxious, or potentially noxious stimuli, there …ncrease in the secretion of adrenocorticotrophic hormone (ACTH) and, consequently … in circulating cortisol or corticosterone (Noble 1950, 1955). Thus, if stress in reindeer …flected in elevated cortisol levels like it is in sheep (Pinaretto and Vickery 1972), cattle …ston and Buckland 1976) and moose (Frantzman *et al.* 1975), the lack of a significant …se in circulating cortisol in Spitzbergen reindeer during winter may indicate that these …als are not under severe thermal or nutritional stress. The profound seasonal changes …rowth hormone levels in Spitzbergen reindeer therefore most likely reflect a specific …bolic adaptation which includes a remarkable fat-storing capacity in periods with …le food supply and a well-developed ability for retrieval of stored body fat during periods …ood scarcity.

…he most …ell known effect of thyroxine is acceleration of metabolic rate measured as …eased oxygen consumption and heat production (Saher 1950), but thyroxine also in…ects behavior. Hyperthyroidism results in nervousness and hyperactivity while hypo…roidism is associated with lethargic behavior (Rawson *et al.* 1955). Reduced feed intake

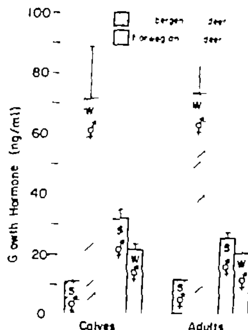


Fig. 1



Fig. 2

Fig. 1 Growth hormone levels in serum from Spitzbergen reindeer collected in August (S) and in March (W). Data are compared to similar results from Norwegian semidomesticated reindeer (Ringberg Lund-Larsen *et al* 1978 a). Each value is given as Mean ± S.E. (Number of animals in brackets)

Fig 2. Cortisol levels in serum from Spitzbergen reindeer collected in August (S) and in March (W). Data are compared to similar results from Norwegian semidomesticated reindeer (Ringberg Lund-Larsen *et al* 1978 b) Each value is given as Mean ± S.E. (Number of animals in brackets)

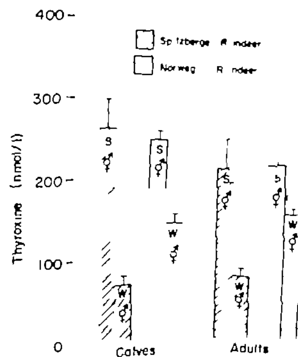


Fig. 3. Thyroxine (T) levels in serum from Spitzbergen reindeer collected in August and in March (W). Data are compared to similar results from Norwegian semidomesticated reindeer (Ringberg Lund-Larsen *et al* 1978 a). Each value is given as Mean ± S.E. (Number of animals in brackets)

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has been shown to reduce the plasma level of thyroxine (T) in white-tailed deer (Se 1972) and in goats (Abdullah and Falconer 1977), and to reduce resting metabolism roe deer (Weiner 1977) In both Norwegian and Spitzbergen reindeer thyroxine is lower in winter than in summer but in the latter the difference is very large. In fact, from the low winter concentrations of thyroxine in Spitzbergen reindeer one would noticeable behavioral changes.

It is well known that Spitzbergen reindeer exhibit a very "tame behavior while human interference during summer elicits a "normal flight reaction. Based on data presented in this report I wish to put forward the hypothesis that the winter ness is caused by the very low thyroxine levels, and is presumably associated with metabolic rates. In the Svalbard-environment devoid of reindeer predators, this "winter dormancy" could be possible as a mode of energy conservation for such insulated animals. The adjustments of lipid metabolism, the lack of stress symptoms the apparent winter dormancy all suggest that the Spitzbergen reindeer are uniquely to their high arctic island habitat

I am indebted to Professor O P Foss, Oslo City Hospital. Ulleval, for performing the thyroxine The skillful technical assistance of chem. ing. V Schuller Jacobsen, Dept. of Physiology Veterinary C of N rw y is also gratefully a knowledged Professor J Krog, Institute of Zoophysiology Univ Osl Norway introduced me to the field of Arctic Biology and supported all stages of the project Logistic assistance on Svalbard was supplied by Professor J Krog and by the Norwegian ... dministration of N A Ordisland. This project was supported by the Norwegian Research Co Science and the Humanities as Project No D 68 70-5, Grant No. GM 10402, NIH, U.S. Public Service and ERDA Contract EY 76-S-06-2229 TA No 3

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-ministering the beta-adrenergic receptor blocker propranolol and the "immobiliza- -tress" response could also be blocked by adrenalectomy An increase in $CMRO_2$ has -een shown to occur during hypoxia (*Berntman et al.* 1977). *In man considerable varia-* n CBF and $CMRO_2$ have been reported when repeated measurements were made in me patients during resting conditions" (Lassen *et al* 1977).

gain further knowledge about the factors affecting CMRO and CBF (*i.e* blood flow nit weight of brain) we have in the present study investigated the effect of CO and the of beta-adrenergic receptor blockage upon these variables. The conditions presently -ed are partly the same as those investigated by Berntman *et al.* (1978) using the Kety idt technique with inhalation of ^{133}Xe for determination of CBF It is apparent from reviously mentioned studies that the resting level" for $CMRO_2$ and CBF may not well defined as originally thought and therefore we have applied a technique which s CBF-measurements at short intervals thus in testing the effect of various agents the imental animal may serve as its own control. This technique also has the advantage of ding more reliable results than the Kety Schmidt technique (Eklöf *et al.* 1973) at the high CBF alues encountered during hypercapnia.

Methods

rats weighing 200–300 g were used The animals had free access to water and food pellets until ion.

rbral blood flow (CBF) and oxygen consumption ($CMRO_2$) were measured using the rapid and re- e method recently developed in our laboratory (Hertz *et al.* 1977) This intraarterial ^{133}Xe injection que was originally described in man by Høedt-Rasmussen *et al.* 1966.

animals were anesthetized with halothane 0 8 in gas mixture of 70 N_2O 30 O_2, trache- mized and artificially ventilated on an animal respirator (Braun, Melsungen). All extracerebral hes from both carotid arteries ere ligated including the pterygopalatine artery A polyethylene ter for injection of ^{133}Xenon was inserted in retrograde fashion into the right external carotid artery ip of the catheter was placed at the carotid bifurcation to avoid obstruction of flow into the internal id A femoral artery as cannulated for continuous recording of mean arterial blood pressure (MABP) for measurement of pHa, $PaCO_2$ and PaO_2 using microelectrodes (Radiometer Copenhagen) A ral vein as cannulated to enable blood transfusion and drug infusion. A small skin incision as made the skull and hrough burr hole in the lambdoid process cannula was inserted for sampling of ral venous blood from the sagittal sinus. Rectal temperature was kept close to 37 C (±0 5°C) by ns of heating blanket MABP was kept within ±10 of the initial value by transfusion of fresh donor d All ounds ere infiltrated with lidocain 1 t avoid pain after switching off halothane

n completion of the surgical preparation hich took 40–45 min, the animals were immobilized with nyl choline 20 mg kg and halothane was switched off

or each measurement of CBF 20 μl bolus of the radioactive, inert gas ^{133}Xenon dissolved in saline mCi, ml, Amersham) as injected into the internal carotid and the washout curve was obtained with mated NaI(Th) crystal (aperture 8 mm) placed over the head ipsilateral to the injection catheter

or determination of $CMRO_2$, arterial and cerebral venous blood was sampled in 25 μl Carlsberg pipettes en 30 after ^{133}Xenon injection and in these blood samples total oxygen concentration was measured g the polarographic method described by Borgström *et al.* (1974).

BF and $CMRO_2$ measurements in the different situations were made in duplicate and the values aver d.

Calculations

F was calculated from the initial slope of semilog plot of the ^{133}Xenon residue clearance curves. The mula used is given by Olesen *et al.* (1971). $rCBF_{init}$ = λ ln 10 D_0 100 (ml/100 g/min) using

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The effect of propranolol on cerebral oxygen consumption and blood flow in the rat measurements during normocapnia and hypercapnia

By

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Abstract

HEMMINGSEN R., M M HERTZ and D I BARRY *The effect of propranolol on cerebral oxygen consumption and blood flow in the rat measurements during normocapnia and hypercapnia* Acta physiol scand 1979 *105* 274–281

The cerebral blood flow (CBF) and cerebral oxygen consumption ($CMRO_2$) in the rat during normocapnia and hypercapnia were investigated by means of the intraarterial ^{133}Xenon injection technique. Measurements were performed during normocapnia and hypercapnia and the effect of propranolol upon CBF and $CMRO_2$ was studied. The CBF technique applied to rat yield reliable results even in high flow situations. A steady state period of only 10–15 s is all that is necessary to obtain the initial slope of the ^{133}Xenon clearance from which CBF is calculated and measurements may be repeated within minutes. Hypercapnia caused an increase in $CMRO_2$ of 35 % which confirms the findings of other investigators. The beta-adrenoceptor blocker propranolol (2 mg per kg i.v.) prevented this increase and could eliminate an increase in $CMRO_2$ already induced; this indicates that CO_2 affects adrenergic mechanisms. Although propranolol eliminated the $CMRO_2$ response to hypercapnia, it only reduced the CBF response; this dissociation of CBF and $CMRO_2$ response occurred probably because the beta-receptor blockage only eliminated the CBF increase mediated through an increased $CMRO_2$ (cellular response) whereas a direct CO_2 effect upon arterioles (vascular response) persisted

It has been generally considered that the cerebral rate of oxygen consumption ($CMRO_2$, "cerebral metabolic rate for oxygen" – ml O_2 100 g brain min) could not increase above the "resting level" found in conscious individuals except during extreme conditions. Such conditions mentioned in the older literature include seizures (Schmidt *et al.* 19[illegible]), extreme anxiety (Kety 1952) and continuous i.v. infusion of epinephrine (King *et al.* 19[illegible]).

Recently however it has been shown that $CMRO_2$ may increase in unanesthetized rats during immobilization, "immobilization stress" (Carlsson *et al.* 1977) and during hypercapnia (Berntman *et al.* 1978). In both situations the $CMRO_2$-increase could be prev[illegible]

* David I. Barry is supported by a Danish Government Scholarship and a Unesco/International Brain Research Organization Travelling Fellowship.



1 Simultaneously recorded ¹³³Xenon wash-out curves which illustrate the reproducibility of CBF-measurements. Measurements are from an animal of group A: double measurements were made 5 min interval during normocapnia, hypercapnia and during hypercapnia after administration of propranolol 2 mg/kg I.V. $PaCO_2$ in mmHg; CBF in ml/100 g/min. Mean arterial blood pressure virtually unchanged.

animals were given propranolol a slight decrease in MABP occurred, but all animals had an MABP above 130 mmHg which is normal for rats of this strain. *Table II* shows values of CBF and $CMRO_2$. *When hypercapnia was induced CBF increased by 2.5 times the normocapnic value* and when propranolol was given CBF decreased to 1.8 times the normocapnic value (cf. Table II). These changes were statistically significant. During hypercapnia $CMRO_2$ increased by 35 % of the normocapnic value and after administration of propranolol CMRO decreased to the normocapnic value, i.e. the significantly higher CMRO existing during hypercapnia was pharmacologically reduced to the initial level.

Group B

Values of $PaCO_2$, PaO, pHa and MABP from this group are given in *Table III*. As in group A hypercapnia was accompanied by the expected decrease in pHa. MABP was kept practically unchanged after induction of hypercapnia. *Table IV* shows CBF and CMRO values. Hypercapnia led *to an increase in CBF by 1.8 times the normocapnic value* whereas no change in CMRO occurred.

Table I. Group A. $PaCO_2$, PaO_2, pH and mean arterial blood pressure (MABP) in rats during normocapnia and during hypercapnia without and with propranolol 2 mg/kg I.V. Median and 25–75 percentiles are given. N = 7

	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	pH	MABP (mmHg)
Normocapnia	36 (34–41)	121 (115–121)	7.40 (7.38–7.40)	150 (147–155)
Hypercapnia	81 (78–82)	129 (124–133)	7.14 (7.10–7.17)	155 (148–160)
	83 (–91)	108 (105–116)	7.09 (7.04–7.12)	136 (132–146)

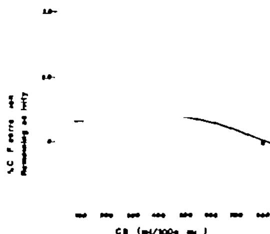


Fig. 1 Curve for correction for remaining activity from previous measurements. The actually measured CBF is found on the abcissa and then the corresponding ordinate-value shall be multiplied by the percentage of remaining activity to yield the correction factor to be added to the measured CBF-value

tissue blood partition coefficient for grey matter $\lambda_g = 0.87$ ml/g. D_0 is the initial slope of the arterial ^{133}Xe non residue clearance curve The slope was obtained from the first 10–15 s of the curve.

A correction for the activity remaining from previous measurements was applied when this activity exceeded 2% of the peak height of the linear washout curve The correction was performed as described by Olesen *et al* (1971) using the graph in Fig. 1 The correction thus rests on the assumption that the remaining activity stays constant during the period required for measurement of the initial slope and that the correction is proportional to the remaining activity when this constitutes 0–10 per cent of the peak activity recorded after the bolus injection. The corrections are therefore valid as long as the CBF of grey matter is several times higher than the CBF of white matter In the present study no measurement was made until the remaining activity was less than 5 of the peak value

$CMRO_2$ was calculated by multiplying the arteriovenous difference in oxygen concentration by the corrected CBF value

Experimental group

3 experimental groups were studied. In each group the hypercapnia was induced by adding CO_2 to the inhaled gas mixture of 70% N_2O 30% O_2. In group A and B propranolol mg/kg diluted in 0.5 ml fresh donor blood was administered slowly over a period of 5 to 8 min (this being necessary to avoid a sudden decrease in MABP).

Group A (7 animals) After immobilization and removal of halothane, the rats were allowed to equilibrate for a period of 20 min before CBF and $CMRO_2$ were measured during normocapnia. Hypercapnia was induced ($PaCO_2$ adjusted to 80 mmHg) and 15–20 min later CBF and $CMRO_2$ were measured again. Still maintaining hypercapnia, propranolol was administered and after 30 min the final CBF and $CMRO_2$ measurements made

Group B (6 animals) In this group of animals propranolol was given immediately after halothane was switched off and the animals had been immobilized. After 30 min of normocapnia CBF and $CMRO_2$ were measured. Hypercapnia was induced and after 15–20 min CBF and $CMRO_2$ were measured again.

Group C (4 animals) After halothane was switched off CBF and $CMRO_2$ were measured in 4 animals after 20 min and 50 min of hypercapnia in order to investigate whether CBF and $CMRO_2$ changed during the intervening period.

Statistical differences were tested by non-parametric methods differences within groups were tested by Wilcoxon's test for paired data whereas differences between groups were tested by Mann-Whitney test for unpaired data.

Results

The method used for measurements of CBF is theoretically and practically well suited for repetitive measurements in both high flow and rather low flow situations (see discussion). The reproducibility of measurements is illustrated in Fig. 2.

Group A

Values of $PaCO_2$, PaO_2, pHa and MABP are given in *Table I* As expected an increase in $PaCO_2$ to about 80 mmHg was accompanied by a decrease in pHa to about 7.1 When the

in). After 30 min of hypercapnia ($PaCO_2$ 89 mmHg) CBF was 430 ml/100 g/min (quartiles 417–448 ml/100 g/min) and $CMRO_2$ was 10.5 ml/100 g/min (quartiles 9.7–12.5 ml/100 g/min). MABP and pHa were unchanged. CBF and $CMRO_2$ were thus unchanged after 30 min of hypercapnia.

Discussion

Since the perfusion coefficient (blood flow per unit weight of tissue) is (normally) far greater in the grey matter than in the white matter and since white matter constitutes only a rather small part of the rat's brain (Nilsson and Siesjö 1976), the major part of the intracarotidly injected ^{133}Xe will initially be located in the grey matter. Consequently the initial slope of the recorded ^{133}Xe clearance curve will predominantly reflect the perfusion coefficient of the grey matter (extracerebral contamination was avoided by ligation of the extracerebral branches of the carotid artery). Venous blood collected from the torcular sinus will mainly be constituted by blood perfusing grey matter and the arterio-venous oxygen concentration difference calculated from the oxygen concentration of such venous blood should be close to the a-v difference for blood perfusing grey matter only. It follows that a product of a calculated a-v difference and the corresponding calculated perfusion coefficient should give the rate of oxygen consumption per unit weight of grey (cortical?) matter with good approximation.

In spite of the close theoretical relationship (Lassen 1976) between the Kety-Schmidt method (modified for rat by Eklöf *et al.* 1973) and the intracarotid injection method there are several reasons rendering the latter method preferable in rat studies: 1) Only a 10–15 s period of constant cerebral blood flow and flow distribution is necessary to obtain the initial slope, and 2) measurements can be repeated within few minutes thus enabling a more dynamic approach to the study of CBF and $CMRO_2$. 3) In the rat very high flow values may occur and in such situations CBF can easily be calculated from the initial slope whereas a calculation according to the Kety-Schmidt principle rests heavily upon determination of one or two venous concentrations within the initial period of desaturation when the concentration changes quite rapidly with time.

The discussion of the CBF and $CMRO_2$ results will be separated in 3 parts:

1) The effect of hypercapnia upon $CMRO_2$,
2) The relation between CBF and $CMRO_2$,
3) "Resting state values" of CBF and $CMRO_2$.

Ad 1) Induction of hypercapnia ($PaCO_2$ about 80 mmHg) resulted in a 35% increase in $CMRO_2$, which is consistent with the 22% difference between normocapnic and hypercapnic rats found by Berntman *et al.* (1978). In an earlier study (Eklöf *et al.* 1973) no increase in $CMRO_2$ was measured but their CBF-technique is not suited for measurement of the very high CBF-values occurring during hypercapnia. In both studies animals were anaesthetized with 70% N_2O/30% O_2.

The CO_2-induced increase in $CMRO_2$ was prevented by the beta-adrenergic receptor blocker propranolol and an increase already induced could be eliminated by propranolol. The decrease in $CMRO_2$ in the hypercapnic animals after administration of propranolol

TABLE II *Group A* Cerebral blood flow (CBF) and cerebral metabolic rate for oxygen ($CMRO_2$) at normocapnia and at hypercapnia without and with propranolol 2 mg/kg I.V. Median 25–75 percentiles are given. N = 7

	CBF (ml/100 g/min)	$CMRO_2$ (ml/100 g min)	Statistics (within group)
Normocapnia (N)	103 (99–135)	13 1 (12.4–14 1)	*CBF* N H p [illegible] N/HP [illegible] H/HP p [illegible]
Hypercapnia (H)	361 (313–461)	17 7 (14 8–19.2)	*CMRO*$_2$ N/H p [illegible] H/HP p<[illegible]
Hypercapnia + propranolol (HP)	278 (224–301)	12.9 (10.1 15.3)	

TABLE III *Group B* $PaCO_2$, PaO_2, pH and mean arterial blood pressure (MABP) in rats during normocapnia and hypercapnia in animals given propranolol 2 mg/kg I V Median and 25–75 percentiles are given. N = 6.

	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	pH	MABP (mmHg)
Normocapnia + propranolol	35 (34–36)	123 (117 126)	7 45 (7 44–7 45)	148 (147 149)
Hypercapnia + propranolol	80 (76–85)	134 (133–137)	7 10 (7 09–7 12)	145 (136–150)

Comparison between group A and B

Both CBF and CMRO during *normocapnia* were lower in the propranolol treated group as compared to the untreated group A ($p < 0\,05$). This was also the case during *hypercapnia* when propranolol treated animals of group B were compared to the still untreated animals of group A. (For CBF $p < 0.05$ for CMRO $0\,05 < p < 0\,10$). The median $CMRO_2$ in untreated, hypercapnic animals of group A was 65 % higher than the corresponding value in the treated group B

There were no statistical differences in CBF or CMRO between hypercapnic animals treated with propranolol before (group A) and after (group B) induction of hypercapnia although the values in group A tended to be higher

Group C

After 20 min of hypercapnia (PaCO ~ 85 mmHg) CBF was 463 ml/100 g/min (quartiles 430–528 ml/100 g/min) and CMRO was 10.2 ml/100 g/min (quartiles 9 4–13 7 ml

TABLE IV *Group B*. Cerebral blood flow (CBF) and cerebral metabolic rate for oxygen ($CMRO_2$) during normocapnia and hypercapnia in animals given propranolol 2 mg/kg I.V. Median 25–75 percentiles are given. N = 6.

	CBF (ml/100 g/min)	$CMRO_2$ (ml/100 g/min)	Statistics (within group)
Normocapnia (N) + propranolol	73 (71 73)	10.8 (10.2 11 3)	CBF N/H $p < 0\,05$
Hypercapnia (H) + propranolol	207 (164–253)	10 7 (9.2 11 3)	$CMRO_2$, ns

n). After 50 min of hypercapnia ($PaCO_2$ = 89 mmHg) CBF was 430 ml/100 g/min (quartiles 417-448 ml/100 g/min) and $CMRO_2$ was 10.5 ml/100 g/min (quartiles 9 7 12.5 ...00 g/min). MABP and pHa were unchanged. CBF and CMRO were thus unchanged ... 50 min of hypercapnia.

Discussion

... the perfusion coefficient (blood flow per unit weight of tissue) is (normally) far greater ... grey matter than in the white matter and since white matter constitutes only a rather ... part of the rat's brain (Nilsson and Siesjö 1976), the major part of the intracarotidly ...ed ^{133}Xe will initially be located in the grey matter. Consequently the initial slope of ...corded ^{133}Xe clearance curve will predominantly reflect the perfusion coefficient of grey matter (extracerebral contamination was avoided by ligation of the extracerebral ...nches of the carotid artery). Venous blood collected from the torcular sinus will mainly be ...stituted by blood perfusing grey matter and the arterio-venous oxygen concentration ...erence calculated from the oxygen concentration of such venous blood should be close ...he a-v difference for blood perfusing grey matter only. It follows that a product of a ...ained a-v difference and the corresponding calculated perfusion coefficient should ... the rate of oxygen consumption per unit weight of grey (cortical?) matter with good ...proximation.

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was not due to a simple time-effect as no decrease was observed in 4 animals (group C) kept hypercapnic for the same period of time without receiving propranolol.

The ability of propranolol to block the CMRO increase during hypercapnia indicates that CO affects adrenergic mechanisms. Adrenalectomy does not prevent the CMRO increase caused by hypercapnia (Berntman *et al* 1978) and hence CO_2 must be assumed to exhibit its CMRO increasing effect centrally The finding that CO increases the desynchronized activity of cerebral cortex as measured by EEG in sleeping man (Bülow 1963) also points at an activating effect of CO on the brain as does the increased rate of formation of monoamines found in the brain during hypercapnia (Prous *et al* 1977 Carlsson *et al* 1977). MacKenzie *et al* (1976) have shown in baboons (normocapnia) that a $CMRO_2$-increase of the same order of magnitude (23 %) can be measured during reserpin induced release of endogenous cerebral norepinephrine, whereas administration of propranolol was followed by a decrease in CMRO as compared to pretreatment value.

It should be emphasized that the effect of CO upon CMRO is by no means fully elucidated In the 4 animals a group C the $CMRO_2$ values observed (during hypercapnia) did not differ significantly from those obtained in animals of group A in the normocapnic period this may indicate interindividual differences in brain reactivity to CO

Ad 2) After administration of propranolol a dissociation between CBF and CMRO response to changing $PaCO_2$ was revealed. Propranolol eliminated the $CMRO_2$-response to hypercapnia whereas the CBF response was only moderately reduced. The latter finding confirms earlier results from baboon (MacKenzie *et al* 1976 Aoyagi *et al.* 1976) and rat (Berntman *et al* 1978)

It seems most probable that the dissociation of CBF and CMRO response to hypercapnia after propranolol was established because the beta receptor blockage only eliminated a CBF increase mediated through an increased CMRO (cellular response) whereas a direct CO effect upon the arterioles (vascular response) persisted this direct effect was probably mediated through an increased hydrogen ion concentration (see Severinghaus and Lassen 1967) The beta-adrenergic receptors responsible for the metabolic CO_2-response are probably localized in the cerebral cortical parenchyma beta-receptors have been shown to exist in this location whereas no beta-receptors were seen in the brain vessel walls of the rat when using a flourescence technique (Melamed *et al* 1977) In relation to this it is important to know that propranolol easily crosses the blood brain barrier (Olesen *et al* 1978).

Ad 3) During resting state conditions" (normocapnia, N O anaesthesia) the propranolol treated animals (group B) had a significantly lower CMRO than had the untreated animals (group A) the $CMRO_2$ just above 10 ml/100 g/min obtained in group B was close to the value obtained by Carlsson *et al* (1977) in propranolol treated unanaesthetised rats ("no mobilization stress") and this may reflect an ability of beta-adrenergic receptor blockade to affect adrenergic "tonus" upon CMRO and hence to diminish interindividual variations in CMRO in unanaesthetised and lightly anaesthetised animals

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...ctile strength (Folkow *et al.* 1971 b). However the relationships between arterial ...re (P_A) and flow (Q), obtained during perfusion with plasma substitute at *maximal* ...tation in the mentioned study gave the impression that the SHR renal resistance ...were structurally somewhat widened compared with the NCR ones, in contrast to ...nation in other circuits. Recently Collis and Vanhoutte (1977), in a similar com... though using a colloid-free perfusate, observed no real difference between the SHR ...NCR renal vascular beds in this latter respect.

...h results concerning total renal resistance are in apparent contrast to recent findings ...ow *et al.* 1977), which strongly suggest a considerable, structurally based increase of ...tio between the pre- and postglomerular resistances in adult SHR, a difference to NCR ...was barely traceable in early borderline hypertensive age. Thus, when P_A was from very ...evels gradually elevated to parallel-perfused maximally vasodilated SHR and NCR ...ys, glomerular filtration (GF) started in SHR first at a P_A level that was 30–40 mmHg ...r than in NCR. Further at still higher P elevations the P – GF relationship for the ...kidneys was throughout shifted some 30–40 mmHg along the P_A abscissa to the right ... NCR ones. Likewise, if urether pressures were equally increased in similarly perfused ... and NCR kidneys, GF ceased at a much lower urether pressure level in SHR than in ... (Gothberg *et al.* 1976). Such findings can be explained only by a considerable increase ...e structurally determined pre/postglomerular resistance ratio in established SHR hyper...on, while glomerular filtration capacity was not yet appreciably reduced to judge from ...parallelity of the P – GF relationships in SHR and NCR (Folkow *et al.* 1977).

...e question arises how such apparently contradictory results concerning total *versus* ...ective-segmental renal resistances may be explained. A clue is given by the observation ...thberg *et al.* 1976) that "tissue pressure" (P_t) was consistently much lower in SHR than ...NCR kidneys at equal P levels, further that P increased far less in the SHR kidneys at ... P increments. It is known that P in kidneys mainly reflects the tubular distension ...fluid, caused by the glomerular filtration. The higher the GF rate, and/or the lower the ...ular reabsorption at given resistance characteristics of the tubular system, the higher the ...age tubular pressure. For such reasons renal P increases will be particularly marked ...filtration is excessive (e.g. at low perfusate colloid osmotic pressures as in Collis and ...houtte's study) or/and when tubular reabsorbtion is greatly reduced (e.g. by perfusate ...ling as in the study by Folkow *et al.* 1977). *Since active preglomerular autoregulation* ...renal flow is eliminated by maximal vasodilatation during such artificial perfusions, P_A ...s are bound to be associated with considerable and largely proportional P elevation. ...ever a particularly strong renal "passive" autoregulation of flow is thereby introduced ...(Waugh 1958, Folkow and Langston 1964) because the true pressure head approximates ... P rather than P alone. A structurally increased pre/postglomerular resistance ratio in ...R then implies that P rises will here be less pronounced than in NCR. In other words, ...true pressure head, P – P is at equal P levels correspondingly higher in SHR than in ...R.

...For such reasons the true renal resistance to flow in SHR and NCR cannot be properly ...lged by the P – Q relationships alone as long as glomerular filtration occurs, implying ...at the results of both Folkow *et al.* (1971 b) and of Collis and Vanhoutte (1977) are in this ...

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Apparent and true vascular resistances to flow in SHR and NCR kidneys as related to the pre/postglomerular resistance ratio

By

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Abstract

GÖTHBERG G., S LUNDIN S E. RICKSTEN and B FOLKOW *Apparent and true vascular resistances to flow in SHR and NCR kidneys as related to the pre/postglomerular resistance ratio* Acta physiol scand 1979 *105* 282 294

In maximally vasodilated SHR and NCR kidneys, perfused with filtrable and non-filtrable ... analyses have been performed concerning the extent of average renal tissue pressure (P) elevation ... occurs upon glomerular filtration and causes passive autoregulation of flow The results ... importance of distinguishing between apparent ($P_A - P_V$/flow) and 'true' ($P_A - P_t$/flow) renal ... flow This is particularly so when the compared SHR and NCR renal vascular beds differ not only ... total renal resistance but also concerning the structurally determined ratio between the pre- and ... glomerular resistances. The combined results show that this ratio is considerably elevated in adult SHR ... neys because of structural vascular changes, which is perhaps the most efficient way of resetting the ... term barostat function of the kidneys in hypertension. It is also illustrated how the altered pre- ... glomerular ratio in SHR in combination with P_t rises can so markedly distort the results of ... precise *in vitro* comparisons of SHR and NCR renal vascular beds as to give entirely misleading ... with underestimations of the structurally based vascular hyperreactivity of the preglomerular ... SHR.

Among systemic circuits the renal vascular bed is of particular interest in hyper... because it affects so intimately the water-salt equilibrium (*cf* Guyton *et al* 1974), b... contributing to the overall increase of resistance. It is known from many studies (*e g* Fo... *et al* 1973 Folkow 1975 Noresson, Hallbäck and Hjalmarsson 1978) that system... sistance vessels almost from the start of hypertension develop a proportional adaptive ... thickening associated with a structural luminal narrowing, a type of structural auto... tion largely confined to the pressure-exposed precapillary resistance vessels where ... as an amplifying and strengthening lever for active changes in smooth muscle ... (Folkow *et al* 1974) Also the renal resistance vessels appear to exhibit a considerable ... thickening in hypertension, to judge from hemodynamic comparisons of perfused k... from SHR and normotensive controls (NCR) and here reflected as an increased ste... of the dose response resistance curve to pressor agents, associated with an elevated ...

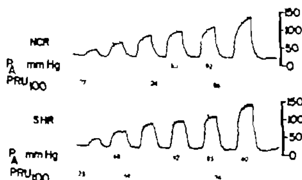
...are closely similar. As measured ...th an Ostwald viscosimeter the viscosity of kerosene was 35 ... than that of 2 ... dextran-Tyrode solution at equal temperatures. Therefore the kerosene flow values ... multiplied by 1.35 in order to provide directly comparable pressure-flow curves for the two perfusates ... respect to their viscosity. These curves then coincided well at pressures below the levels ... here glomeru... ...tion starts.

...me experiments the approximate intrarenal "tissue pressure" was measured in the dextran-Tyrode ... kidneys by ...ay of cannulas of 0.3 mm diameter which were inserted to identical depths into the ...rtex. It should be realized, however, that such local pressures ...ll only exceptionally be representa... the true average intrarenal pressure level, and were therefore mainly used for general orientation ...e events.

Results

...arterial pressures in the awake NCR and SHR differed about 30 per cent, this dif... being slightly lower (about 23 per cent) in the subgroup used for kerosene perfusions, ... because NCR here showed slightly higher pressures than is usual for NCR.

...dextran-Tyrode perfused kidneys immediate increases of renal flow from a low ...gh, constantly held level caused a two-phasic P... increase: first an initial prompt rise, ...ed by a second phase were P... increased progressively and gradually levelled off after ...s (Fig. 1). This second phase of P_A rise was always, as illustrated in this Figure, more ...unced in NCR than in SHR. As flow was then constant, the second phase of the P_A ...se reflects a progressive increase of resistance which, however, cannot represent any ...d autoregulatory response since the vessels remained completely relaxed due to high ...ntrations of vasodilator agents and the low tissue temperature. I... this situation a ...y proportional increase of glomerular filtration (GF) will occur progressively dis... ...ng the tubules and increasing pressure within them. Some accumulation of fluid might ... also in the renal interstitium in case filtration occurs across the tubular capillaries as



...1. Changes in arterial pressure (P_A) during paced perfusion ...th dextran-Tyrode solution ...t different ...rates of maximally vasodilated, decapsulated kidneys from 3–4 month old SHR and NCR. When ...ere increases in flow are induced, here lasting only 10 sec, there is ...two-phasic P... elevation: an initial ...phase ...th the sudden flow increase followed by ...more gradual phase that usually levels off first ...15–30 s. As flow ...constant during this second phase of P_A rise, it reflects ...progressive increase of ...resistance ...thin the kidneys, ...hich must be ascribed to passive phenomena since the renal vessels ...re fully relaxed and unresponsive ("pseudo autoregulation"). The vertical dotted line roughly separates ...phases and the deduced figures for renal resistance ...flow given as PRU values, therefore ap...ately reflects the truly ...ascular resistances present before passive autoregulation sets in. Note that ...ation of passive autoregulation is more pronounced in NCR than in SHR.

particular respect misleading. The presence of passive autoregulation will overrate renal resistances in both NCR and SHR and particularly so in the NCR kidneys in proportion to their lower pre/postglomerular resistance ratio. The aim of the present study was therefore to find out the true levels of total vascular resistance proper in maximally dilated SHR and NCR kidneys, thereby revealing also the extent to which P increases the P_A-Q relationships during artificial perfusion and glomerular filtration. From these data and those by Folkow *et al* (1977) it would be possible to deduce more precisely the pre/postglomerular resistance ratio as well as the postglomerular resistance when the SHR and NCR renal vascular beds are determined only by their structural dimensions. First then the nature, extent and hemodynamic significance of the vascular adaptation occurring in the SHR kidneys along with the development of hypertension can be fully understood It should here be emphasized that a sustained increase in the pre/postglomerular resistance ratio represents perhaps the most efficient way of resetting the renal long term barostat function in hypertension (*cf* Guyton *et al*. 1974)

Methods

20 matched pairs of male SHR and NCR at the age of 3–4 months were used. During light ether anesthesia the caudal artery of the two animals was cannulated with polyethylene catheters and mean arterial pressure (P_A) was measured after recovery from their anesthesia. Final anesthesia for the surgical preparation was induced by an intra-arterial infusion of Nembutal, 3–4 mg/100 g b w

In identical and largely simultaneous preparations a pair of SHR and NCR was exposed of the abdominal aorta was freed for about 10 mm distal and 5 mm proximal to the renal arteries, great care being taken to ligate all branches along this aortic section except the two renal arteries and the mesenteric artery In both animals the kidneys were then cautiously decapsulated and a catheter, directed towards the renal arteries, was inserted in the distal aortic part after ligation of the proximal part of the aorta. Perfusion of the two kidneys with oxygenated per cent dextran (Macrodex) in Tyrode solution was immediately started in both animals. The animals were killed and the proximal end of the mesenteric artery cannulated for P_A recording during the artificial renal perfusion in both animals. Once this procedure was going, also the proximal aortic end was cannulated between the proximal ligature and the right renal artery and through this catheter the right kidneys of both animals could be perfused separately with mineral oil kerosene after that a temporary ligature had been placed around the aorta between the two renal arteries. Thus, the right kidneys of the two animals were perfused in parallel with mineral oil and the left kidneys also in parallel, with oxygenated Tyrode solution containing 2% dextran.

To secure maximal vasodilatation nitroprusside sodium was dissolved in the 2% dextran-Tyrode solution, to give 0.8 mMol concentration As the kerosene by itself relaxes the vasculature completely according to Waugh, no vasodilating agent was used for the right kidneys. Both perfusates were kept at room temperature (20°C) to minimize active tubular reabsorbtion in the two left kidneys (*cf* Folkow *et al* 19 The mineral oil kerosene earlier used by Waugh (1958) for determining the true vascular resistance in kidneys, is for such purposes suitable as it stays strictly within the renal vascular bed. Thereby glomerular filtration is eliminated, and with that largely the whole passive autoregulatory process in the right SHR and NCR kidneys, while the left ones will have their pressure-flow relations affected by passive autoregulation.

The pressure-flow relationship of the oil perfused kidneys was determined by repeated shifts in flow over a wide range during about 15 min The same procedure was then performed concerning the dextran-Tyrode perfused kidneys of the two animals after that the right renal artery had been ligated, the temporary aortic ligature between the renal arteries removed and the proximal catheter clamped.

The flow values both in the kerosene perfused right kidneys and the dextran Tyrode perfused left kidneys were then calculated per g dry weight of the dextran-Tyrode perfused kidney as the dry weights of the kerosene-perfused kidneys are unreliable because the intravascular oil contents may not be completely eliminated in the heating chamber The dry weights are for this purpose preferable since the dextran-Tyrode perfusion causes some renal edema which may differently affect the NCR and SHR kidneys. Earlier tests on fully intact SHR and NCR kidneys have shown that their relationships between et and

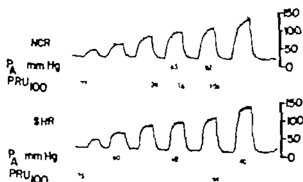
-y clearly similar. As measured [w]ith an Ostwald viscosimeter the viscosity of kerosene was 35 [per cent] [th]an that of 2 dextran-Tyrode solution at equal temperatures. Therefore the kerosene flow values [were mul]tiplied by 1.35 in order to provide directly comparable pressure-flow curves for the two perfusates [corr]ect to their viscosity. These curves then coincided well [a]t pressures below the levels [w]here glomeru[lar filtratio]n starts.

[In the]se experiments the approximate intrarenal "tissue pressure" was measured in the dextran-Tyrode [perfused] kidneys by way of cannulas of 0.3 mm diameter which were inserted to identical depths into the [cor]tex. It should be realized, however, that such local pressures will only exceptionally be representa[tive of t]he true average intrarenal pressure level, and were therefore mainly used for general orientation [of] events.

Results

[A]rterial pressures in the awake NCR and SHR differed about 30 per cent, this dif[ference] being slightly lower (about 23 per cent) in the subgroup used for kerosene perfusions, [] because NCR here showed slightly higher pressures than is usual for NCR.

[In th]e dextran-Tyrode perfused kidneys immediate increases of renal flow from a low [...], constantly held level caused a two-phasic P_A increase; first an initial prompt rise, [followe]d by a second phase were P_A increased progressively and gradually levelled off after [...] (Fig. 1). This second phase of P_A rise was always, as illustrated in this Figure, more [pronou]nced in NCR than in SHR. As flow was then constant, the second phase of the P_A [ris]e reflects a progressive increase of resistance which, however cannot represent any [...] autoregulatory response since the vessels remained completely relaxed due to high [concen]trations of vasodilator agents and the low tissue temperature. In this situation a [...] proportional increase of glomerular filtration (GF) will occur progressively dis[tending] the tubules and increasing pressure within them. Some accumulation of fluid might [occur] also in the renal interstitium in case filtration occurs across the tubular capillaries as



[Fig. 1.] Changes in arterial pressure (P_A) during paired perfusion with dextran-Tyrode solution at differen[t le]vels of maximally vasodilated, decapsulated kidneys from 3–4 month old SHR and NCR. When [step] increases in flow are induced, here lasting only 10 sec, there is [a] two-phasic P_A elevation; an initial [prompt] phase [wi]th the sudden flow increase followed by [a] more gradual phase that usually levels off first [after] 3–10 [sec]. As flow is constant during this second phase of P_A rise, it reflects [a] progressive increase of [re]sistance within the kidneys, [wh]ich must be ascribed to passive phenomena since the renal vessels [are] [max]imally relaxed and unresponsive ("passive autoregulation"). The vertical dotted line roughly separates [the two] phases and the deduced figures for renal resistance to flow given as PRU values, therefore ap[proxim]ately reflects the truly vascular resistances present before passive autoregulation acts in. Note that [the de]gree of passive autoregulation is more pronounced in NCR than in SHR.

particular respect misleading. The presence of passive autoregulation will [illegible] renal resistances in both NCR and SHR, and particularly so in the NCR kidneys [illegible] portion to their lower pre/postglomerular resistance ratio. The aim of the present [illegible] therefore to find out the true levels of total vascular resistance proper in [illegible] dilated SHR and NCR kidneys, thereby revealing also the extent to which P [illegible] the $P_A - Q$ relationships during artificial perfusion and glomerular filtration. For data and those by Folkow *et al* (1977) it would be possible to deduce more [illegible] the pre/postglomerular resistance ratio, as well as the postglomerular resistance [illegible] when the SHR and NCR renal vascular beds are determined only by their [illegible] dimensions. First then the nature extent and hemodynamic significance of the [illegible] vascular adaptation occurring in the SHR kidneys along with the development of [illegible] tension can be fully understood It should here be emphasized that a sustained [illegible] the pre/postglomerular resistance ratio represents perhaps the most efficient way of [illegible] the renal long-term barostat function in hypertension (*cf* Guyton *et al* 1974).

Methods

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To secure maximal vasodilatation nitroprusside sodium was dissolved in the 2 [illegible] dextran-Tyrode [illegible] tion, to give a 0.8 mMol concentration. As the kerosene by itself relaxes the [illegible] cording to Waugh no vasodilating age t was used f r the right kidneys. Both perfusates were kept [illegible] temperature (20°C) to minimize active tubula reabsorbtion in the two left kidneys (*cf* Folkow *et al* [illegible] The mineral oil kerosene earlier used by Wa gh (1958) for determining the true vascular resistance [illegible] kidneys, is for such purposes suit ble as it stays strictly within the renal vascular bed. Thereby [illegible] filtration is eliminated and with that largely the whole passive autoregulatory process in the right [illegible] NCR kidneys, while the left ones will ha e their pressure-flow relations affected by [illegible]

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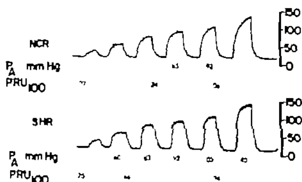
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... Changes in arterial pressure (P) during paired perfusion with dextran-Tyrode solution at different ... of maximally ... modilated, decapsulated kidneys from 3–4 month old SHR and NCR. When ... increases in flow are induced, here lasting only 10 sec, there is ... two-phasic P_A elevation; an initial ... phase ... ith the sudden flow increase followed by ... more gradual phase that ... usually levels off first ... 5–10 ... As flow is constant during this second phase of P_A rise, it reflects ... progressive increase of ... resistance ... rthin the kidneys, ... hich must be ascribed to passive phenomena since the renal vessels ... fully relaxed and unresponsive ("passive autoregulation"). The vertical dotted lines roughly separates ... phases and the deduced figures for renal resistance to flow, given as PRU values, therefore ap... ately reflects the truly vascular resistances present before passive autoregulation sets in. Note that ... of passive autoregulation is more pronounced in NCR than in SHR.

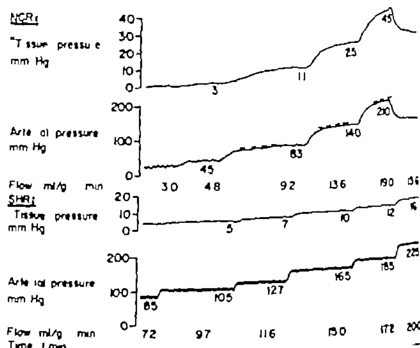
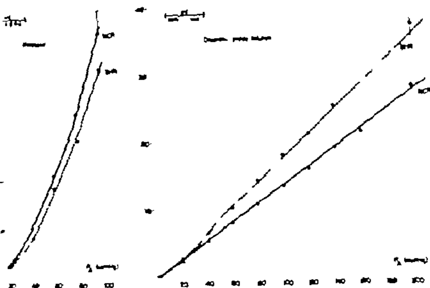


Fig. 2. Recordings of arterial pressure (P_A) and intrarenal tissue pressure (P_t), measured in thin cannulas inserted at identical cortical sites in pair-perfused decapsulated SHR and NCR kidneys, otherwise arranged as described in Fig. 1. This type of P_t recording, reflecting mainly the tubular pressure at the cannula site, shows semiquantitatively how P_t changes with P_A, though it does not necessarily reflect the average P_t level within the whole organ. However, the recordings serve to illustrate how P_t changes were consistently much smaller in SHR than in NCR for given changes in P_A.

Consequently the renal tissue pressure P_t will increase and largely in proportion to the rise in intratubular pressure. This tends to increasingly compress the low-pressure part of the renal vasculature, hence causing a progressive rise in renal resistance, which in reality is rather due to the fact that the true pressure head for vascular perfusion equals $P_A - P_t$. This passive autoregulatory influence of a P_t rise, which mainly depends on the enhanced GF and tubular distension as P_A increases, thus produces an apparent increase in total renal vascular resistance that will not appear in case transcapillary fluid passage be hindered. In some experiments, where P_t was estimated by thin cannulas inserted in the parenchyme of the SHR and NCR kidneys in as identical portions as possible, it was observed that stepwise P_A increases always produced greater P_t increases in NCR, suggesting more pronounced GF increases in the normotensive kidney for given P_A increments (Fig. 2).

However this mode of estimating local intrarenal P_t may not with any accuracy reflect the average P_t changes within the kidney and their precise interference with renal blood flow and therefore it provides only semiquantitative information. In order to better quantify the differences in extent of passive autoregulation in the SHR and NCR kidneys, the P_A-Q relationships were determined over a wide range where flow/g wet kidney weight was plotted against the 'passively autoregulated' P_A levels for both SHR and NCR (Fig. 1, right part). It is seen from the Figure that the apparent total renal resistance to flow is around 25 per cent higher in the NCR kidneys at all P_A levels high enough to allow glomerular filtration. These P_A-Q relationships should be compared with those obtained



Left panel: 7 pairs of decapsulated, maximally modulated kidneys from 3–4 month old SHR and perfused with the non-filtrable material oil kerosene at room temperature, thus eliminating both active and passive autoregulatory processes (Waugh 1958). Note the convexity towards the pressure axis reflecting the characteristic distensibility of resistance vessels when fully relaxed. Note further that the fully dilated, truly vascular resistance to flow is moderately higher in SHR per g of renal tissue, the difference from NCR being significant (p < 0.05) at higher pressure levels.

Right panel: Left kidneys from the same pair of SHR and NCR but here perfused with 2 % Dextran in solution at room temperature and during maximal vasodilatation. Here glomerular filtration starts at low P_A levels and since reabsorption is minimized by cooling, renal tissue pressure rises rapidly with P_A elevations. The P values given represent those reached when "passive autoregulation" is fully developed. Since the viscosity of kerosene is 35 % higher than that of 2 % dextran-Tyrode solution, the values in the two panels are corrected for this difference. Note how the pressure-flow curves for the dextran perfusate are greatly depressed towards the pressure axis once filtration starts, further that the SHR and NCR curves are here reversed.

...g kerosene perfusion where passive autoregulatory influences caused by transcapillary ...r transfer are eliminated. The P-flow relationships for kerosene are presented in the left ... of Fig. 3, but corrected for the difference in viscosity between kerosene and dextran-...de solution. It is then revealed that the true vascular resistance to flow is slightly higher ...nd 15 per cent) in the SHR kidneys than in the NCR ones. Moreover the P_A-Q ...onships are now characteristically convex towards the pressure axis, which is typical ...sistance vessels in general when both active and passive autoregulatory phenomena are ...inated, thus revealing their wall distensibility (Folkow and Löfving 1956, Hallbäck, ...gren and Weiss 1974).

...suming that the vascular beds of the right and left kidneys have, on an average, equal ...ensions in both NCR and SHR, the two sets of P-flow relationships in the left and right ...s of Fig. 3 can be used to deduce the increases in P along with P_A elevations (for the ...R and NCR kidneys, when plotted together (Fig. 4 left part). These P increases are of ...at interest since they approximately reflect the extent of glomerular filtration in the two

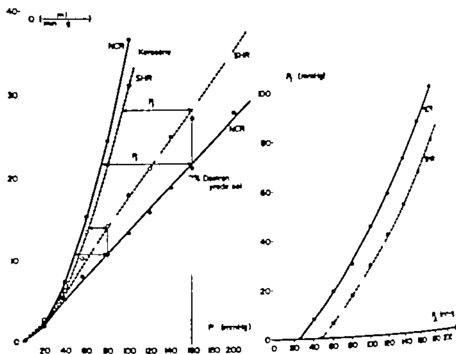


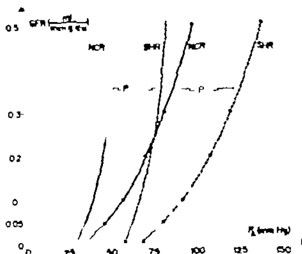
Fig. 4 *Left panel* The two pairs of pressure-flow curves from Fig. 3 are here plotted on the same scale for deductions of the average changes in renal 'tissue-pressure' (P_t) from the difference in P_A at per... with dextran-Tyrode and kerosene as perfusates.

Right panel The deduced P_t elevation for given increases in P_A which are always higher in NCR kidneys than in SHR ones suggesting higher pre/postglomerular resistance ratio in SHR. It can hardly be a reduced glomerular filtration capacity in SHR since the $P_A - P_t$ relationships should then not be...

kidneys, if it is assumed that intratubular resistance to flow and degree of tubular ... tion are largely equal in both. As the results by Folkow *et al* (1977) suggest largely ... glomerular filtration capacities in the NCR and SHR kidneys at this stage of life, the differences in deduced P increases shown in Fig. 4 right part, will fairly closely reflect differences in effective glomerular filtration pressure and filtrate volumes between the SHR and NCR kidneys at given P_A levels. It should be noted how closely these curves of P_A and P_t, resemble the P_A − GFR curves shown in Fig. 1 of the paper by Folkow *et al* (1977), as would be expected since they reflect largely the same processes, though ... and deduced in entirely different way

Discussion

The present experiments illustrate the many difficulties involved when quantitative ... dynamic analyses of the renal vascular bed are performed. If for example, the consequences of an altered vascular design are explored during maximal vasodilatation and ... perfusion, this also eliminates the active preglomerular autoregulation which normally keeps glomerular filtration pressure almost constant. It introduces, in return, ... flow autoregulation, a consequence of the increases in renal tissue pressure (P_t) that ... upon enhanced glomerular filtration and tubular distension in connection with elevat...



...he relationship between P and GFR (ml/min g dry weight) in artificially perfused isolated ...from 3-4 month old SHR and NCR, as earlier presented by Folkow *et al.* (1977), but here con... ...r P increases according to Fig. 4. This serves to illustrate the relationship between the 'true' per... ...ressure for the renal vascular bed (P_A P) and glomerular filtration rate in SHR and NCR kid... ...ws how this correction makes the curves much steeper though still with marked rightward shift ...HR curve in way which can only be ascribed to structurally based elevation of the pre/post... ...lar resistance ratio

...n arterial pressure (P_A) (*cf.* Aukland 1976). Since the effective perfusion pressure for ...al vascular bed equals P P_t, rather than P minus extrarenal P_V, the P increases ...e especially pronounced, and hence hemodynamically important, whenever glomerular ...on is much enhanced or/and tubular reabsorption depressed.

...such reasons comparative analyses of renal vascular resistances can be very mis... ...g if based only on P_A and flow measurements, particularly if the kidneys differ also ...rent pre/postglomerular resistance ratio (Rpre/Rpost), glomerular filtration capacity ...d tubular reabsorption. The influence of such factors on tubular pressure and hence ... can be fully appreciated concerning overall renal hemodynamics only if two different ...sates are used for the comparison, one which is filtered in the glomeruli and one which ...ble to pass across capillary walls, like the mineral oil kerosene (Waugh 1958). With the ...type of perfusate the only possible contribution to 'passive' autoregulation via P ...ases would be if such marked intrarenal vascular distension occurs during P elevation ...P is thereby increased significantly. This approach was utilized in the present com...on of the NCR and SHR renal vascular beds. It should be stressed that the same prin... difficulties would be encountered if these problems were instead studied by micro...ture techniques, and the present 'whole organ' technique has here the great advantage ...ematically averaging pressures, flow and other events for the entire population of ...rons and their blood supply.

...ig. 3 makes it clear that the true vascular resistance to flow (P P_t)/Q is moderately ...her in SHR than in NCR of early adult age, while the reverse is true for the apparent ...stance to flow (P_A P)/Q. In other words, the P increases with P elevations must be

much more pronounced in NCR than in SHR, which is also illustrated by direct measurements shown in Fig. 2. These findings lend further support to earlier (Göthberg *et al* 1976 Folkow *et al* 1977) which strongly suggest the presence of a ally based increase of Rpre/Rpost in early established SHR hypertension. further indicated close similarities between SHR and NCR concerning glomerular capacities and extent of tubular reabsorption in the prevailing experimental ferences in these latter respects could otherwise have resulted in different P levels P_A since they also affect the degree of tubular distension.

Based on the deduced $P_A - P_t$ relationships, shown in Fig. 4 it is possible approximate corrections for the P_t changes occurring when P_A and GFR were each other in adult SHR and NCR kidneys in the experiments by Folkow *et al* (19 seems justified as the same perfusate and perfusion technique was used in the and the animals used were of the same age (3–4 months) and belonged to the same stock. By this P_t correction the more relevant relationship between the true pressure for renal perfusion ($P_A - P_t$) and GFR is obtained and these deduced relationships illustrated in Fig. 5 for adult SHR and NCR. As would be expected, they show GFR increases with arterial pressure rises when the true rather than the 'apparent' fusion pressure is given along the abscissa, but still the SHR curve is almost parallel placed some 25 mmHg to the right of the NCR one along the pressure rise.

This circumstance must imply that for equal arterial pressures the glomerular pressure is 25–30 mmHg lower in adult SHR than in NCR, even when the renal vessels completely relaxed In the same situation the true total renal vascular resistance is per cent higher in SHR to judge from Fig. 3 These data allow for approximate of the structurally determined pre and postglomerular resistance in SHR and NCR respect to their relative differences. If in this situation total renal resistance in NCR arbitrarily set to 100 and Rpre/Rpost to the relatively low level of 25/75 since the of active preglomerular autoregulation would reduce Rpre, the corresponding SHR would be about 120 and 60/60 respectively at an MAP level of 100 mmHg The only chance for the SHR kidney to maintain the same glomerular filtration pressure the NCR one when perfused at 100 mmHg, would be to increase P_A up towards 150 for SHR which largely corresponds to the arterial pressure levels in the awake animal

In other words, the renal barostat function is in SHR most efficiently 'reset' to mean arterial pressure equilibrium by principally the same process of vascular adaptation which is responsible for *structural autoregulation* in other systemic circuits (Folkow 1974 Folkow 1975) It further seems as if the postglomerular resistance in the SHR is, if anything, slightly reduced compared with NCR. This may not be surprising with a high preglomerular resistance, which *in vivo* is reinforced by active autoregulation and vasoconstrictor fibre influences, the mean transmural pressure along the postglomerular vascular sections may well be on average kept somewhat lowered in SHR compared NCR despite the higher arterial pressure level in SHR Since vascular design changed in the opposite direction if local distending pressure is kept subnormal *et al* 1971 a), a modest reduction in the structurally set postglomerular r then be expected contributing to the elevation of Rpre/R R

Further the structural increase of Rpre/Rpost in SHR seems to be associated with a thickened media of preferentially the preglomerular vessels. This is suggested by the characteristic hyperreactivity and increased maximal contractile strength shown by the SHR renal resistance vessels (Folkow *et al.* 1971 b), again a close analogue to the situation for the precapillary resistance vessels in other systemic circuits. It is known that the preglomerular resistance vessels are the major site for 'active' renal autoregulation and also for vasoconstrictor fibre adjustments. Such local and remote excitatory influences on the preglomerular resistance would, as mentioned, tend to further accentuate the elevated Rpre/Rpost in SHR because a structurally increased w/r of the preglomerular vessels implies a potentiation of luminal reduction even if the extent of smooth muscle activation is the same.

On the whole, the *per se* normal principle of rapid structural adaptation of heart and vessels to average changes in local load, shared by almost all tissues, has a series of most important consequences for the induction and maintenance of hypertension. Besides causing early 'resetting' of systemic precapillary resistance (Folkow *et al.* 1973, Folkow 1975), the Frank Starling relationship for the left ventricle (Hallbäck 1975) and of the arterial and cardiac receptors (*cf.* Thorén 1978), such changes will in SHR primary hypertension also efficiently reset also the 'long-term barostat function' of the kidneys. This is evidently achieved by means of a structurally based elevation of the pre/postglomerular resistance ratio, in association with a preglomerular hyperreactivity that is mainly a consequence of a structural w/r increase of these vessels, where the presence of a media thickening is well documented for the renal vascular bed in hypertension also in man (*e.g.* Furuyama 1962). To maintain glomerular filtration unchanged, mean arterial pressure must be kept correspondingly elevated in SHR and, should the filtration capacity later become reduced, arterial pressure must be even further elevated where perhaps a postglomerular constriction in such later stages may contribute.

The present experiments further illustrate how the hemodynamic techniques needed for such quantitative analyses of the renal vascular bed also introduce often marked elevations of renal tissue pressure (P_t), and in a way which tends to mask the true vascular differences between SHR and NCR. This source of error was not fully realized in the initial study dealing with renal hemodynamics in SHR (Folkow *et al.* 1971 b). Here SHR and NCR kidneys were perfused in parallel, with Tyrode solution containing as colloid 3 per cent dextran, and the P_t increases must have affected mainly the estimations of total flow resistance at maximal dilatation, thus overestimating that in NCR and underestimating that in SHR. Such disturbances must even more seriously have affected the similar more recent findings by Collis and Vanhoutte (1977) because the total lack of colloids in the perfusate used by these investigators must here have resulted in marked glomerular filtration even at low P_A levels.

The approximate extent of P_t influences in these two *in vitro* studies may be illustrated by the following comparison to the normal *in vivo* situation. At 100 mmHg pressure level and during maximal vasodilatation, renal blood flow in normotensive rats is around 8 ml/g · min. Also here glomerular filtration increases during vasodilatation but the intact tubular reabsorption tends to keep the P_t increase relatively modest. Effective viscosity of blood flow at normal hematocrit is about 2.3-2.5 cP at high flow rates (Djojosugito *et al.* 1970),

which would largely apply also for the kidneys. An extrapolation to 100 mmHg of the pressure-flow relationships for NCR kidneys during *in vitro* Ficoll-Tyrode perfusion (Folkow *et al* 1971 b) would here mean a flow around 8–10 ml/g min, but at a perfusate viscosity of only around 1–1.2 cP In Collis and Vanhoutte s (1977) renal perfusions with Tyrode solution with a perfusate viscosity about 0.7 cP flow was only some 5-6 ml/g min in the NCR as well as the SHR kidneys at a P_A level of 100 mmHg. In both these studies great care was taken to keep the renal vessels completely relaxed, but correcting for differences in perfusate viscosity reveal that the apparent renal resistance to flow i.e. P_A/Q was nevertheless about doubled in the study of Folkow *et al* (1971 b) and increased some four times in that of Collis and Vanhoutte (1977), compared with rat kidneys in vivo

Clearly the P increases must have been formidable, particularly in the latter study where the lack of perfusate colloids eliminated all restrictions on glomerular filtration and hence on P_t elevations and probably also meant filtration across the tubular capillaries In fact the true pressure head $(P_A - P_t)$ driving the renal perfusion in Collis and Vanhoutte experiments at e g 100 mmHg, could only have amounted to some 40 per cent of that when also renal vascular distensibility is taken into account as judged from the results Fig. 3 left part. If then the Rpre/Rpost is initially higher in the SHR kidneys, as discussed above, P levels must have been correspondingly lower in the SHR kidneys compared with the NCR ones. In other words, the largely equal apparent vascular resistance (P_A/Q) in the SHR and NCR kidneys, observed by Collis and Vanhoutte, must in reality have implied far lower true renal vascular resistance, $(P_A - P)/Q$ but where that in SHR was higher than in NCR largely in proportion to the difference in Rpre/Rpost.

Obviously without knowledge of average P levels total renal vascular resistances cannot be properly compared in SHR and NCR in artificial perfusions, since the presence of a structural Rpre/Rpost increase in SHR inevitably leads to an underestimation of the vascular resistance, and largely in proportion to the increase of Rpre/Rpost. Similar errors are involved in the study by Folkow *et al* (1971 a), though being here more modest in extent, partly because P_A levels were kept lower partly because glomerular filtration was kept limited by the presence of perfusate colloid.

Moreover during tests for renal vascular smooth muscle constriction to nerve stimulation or to constrictor drug infusions, Collis and Vanhoutte used constant optimal perfusion which evidently corresponded to 4–5 ml/g min, where already a major P influence was at hand Again, these pressor responses must have been markedly distorted by these high P levels, and to different extents in SHR and NCR. These high but different P levels would be further accentuated whenever vasoconstrictions during constant flow perfusion included also the postglomerular resistance vessels, as such constrictions further enhance filtration and hence P At given active P_A elevations, the presence of an accentuated preglomerular response in SHR would therefore always imply a relatively smaller P contribution to the P_A rise than in NCR.

Consequently the phenomenon of passive autoregulation via P changes will in these types of comparative analyses tend to greatly reduce the real differences between SHR and NCR concerning the extent of active true vascular responses, and then underestimate the SHR responses more or less in proportion to the higher Rpre/Rpost These distortions may

been so marked in Collis and Vanhoutte's experiments as to largely mask even subtal differences between SHR and NCR kidneys with respect to Rpre/Rpost and premder vascular reactivity

ain, such sources of error would in principle be involved also in the vascular reactivity earlier performed on SHR and NCR kidneys by Folkow *et al.* (1971 b), though being of much smaller impact. The reason is that flow and P_A were here kept much lower rt lth, and the colloid content in the perfusate tended to restrict glomerular filtration ases and hence also P increases. Thus, even though P also here tended to mask the differences in vascular reactivity between SHR and NCR, it was nevertheless evident these results that the SHR renal resistance vessels had a much higher maximal contile strength and produced steeper resistance increases. Such a combination of vascular ners cannot be primarily ascribed to alterations in smooth muscle sensitivity or in nor-agonist characteristics, since they occurred also when the contractile proteins were tly engaged by barium ions, like calcium ions bypassing membrane events and the ro-mechanical coupling mechanisms. They can only be ascribed to the presence of an eased bulk of contractile tissue per unit vessel radius which, of course, by no means ex es concomitant deviations also in the functional characteristics of the vascular effector t.

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which would largely apply also for the kidneys. An extrapolation to 100 mmHg of the pressure-flow relationships for NCR kidneys during *in vitro* Ficoll-Tyrode perfusion (Folkow *et al* 1971 b) would here mean a flow around 8–10 ml/g min, but at a perfusate viscosity of only around 1–1.4 cP In Collis and Vanhoutte s (1977) renal perfusions, [illegible] Tyrode solution with a perfusate viscosity about 0.7 cP flow was only some 5–6 ml/g [illegible] in the NCR as well as the SHR kidneys at a P_A level of 100 mmHg. In both these studies great care was taken to keep the renal vessels completely relaxed, but corrections for the differences in perfusate viscosity reveal that the apparent renal resistance to flow (P_A/Q) was nevertheless about doubled in the study of Folkow *et al* (1971 b) and increased [illegible] four times in that of Collis and Vanhoutte (1977), compared with rat kidneys *in vivo*

Clearly the P increases must have been formidable, particularly in the latter [illegible] study where the lack of perfusate colloids eliminated all restrictions on glomerular filtration and hence on P_t elevations and probably also meant filtration across the tubular capillaries. In fact the true pressure head ($P_A - P$), driving the renal perfusion in Collis and Vanhoutte's experiments at *e.g.* 100 mmHg, could only have amounted to some 40 per cent of this level when also renal vascular distensibility is taken into account, as judged from the [illegible] Fig. 3 left part If then the Rpre/Rpost is initially higher in the SHR kidneys, as described above, P_t levels must have been correspondingly lower in the SHR kidneys compared with the NCR ones. In other words, the largely equal apparent vascular resistance (P_A/Q) in the SHR and NCR kidneys, observed by Collis and Vanhoutte, must in reality have implied far lower true renal vascular resistance, $(P_A - P_t)/Q$ but where that in SHR is higher than in NCR largely in proportion to the difference in Rpre/Rpost.

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Methods

Experiments were performed on 15 normotensive adult male Wistar rats (weighing 320-500 g, age ... weeks). In some animals the awake mean arterial pressure (MAP) was recorded via a PE 50 catheter inserted into the tail artery under light ether anesthesia. The animals were then anesthetized with pentobarbital (Nembutal® 40-50 mg/kg b w) i.a. or i p. The anestesia was maintained by ... pentobarbital in repeated injections i.a. The trachea was cannulated with a PE 200 tube and positive pressure ventilation with pure oxygen was induced with a frequency of 28 per minute and end-expiratory pressure of 12 to 15 cm water. In preliminary experiments (2 rats) this type of artificial ventilation gave ... values for arterial PO_2 (350-450 mmHg) and PCO_2 (35-45 mmHg). To prevent acidosis ... $NaHCO_3$ (0.1-0.2 mM) were injected at intervals. Muscle movements were prevented by gallamine (Flaxedil® 4-5 mg/kg b w) in repeated i.v. injections. Rectal temperature was measured ... and maintained at 37-38°C by means of a thermostatically controlled shielded heat pad.

Surgical procedures

The vagus nerve and the carotid artery were dissected free in the neck either on the right (12 rats) ... side (3 rats). The thorax was opened via a bilateral, intercostal, transternal incision. The pericardium ... split and snares were placed around the ascending aorta and the inferior caval vein near the heart.

A thin catheter (PE 10) was placed with its tip in the left atrium via the right middle lung vein and connected to an ultra low displacement transducer (Gaeltec 3 EA). The right upper and middle lung ... were then removed. The arterial blood pressure was recorded in the tail artery. A jugular vein was cannulated. The vagal trunk was identified just above the lung root and cut in order to eliminate ... activity from the lungs and abdominal viscera.

Recordings

The right or left vagus were cut centrally and placed on a dissection plate. The plate ... constructed by the surrounding muscles and skin and filled with body warm mineral oil. The nerve ... was carefully dissected away by means of sharp forceps under a binocular Zeiss dissection ... Thin filaments were then obtained and placed on a thin bipolar silver electrode and connected to a ... probe (HIP 511E) and the nerve spikes were amplified (Grass amplifier P511).

The high frequency cut off was normally set at 1 000 Hz and the low frequency cut off at ... The output from the amplifier was displayed both on an oscilloscope (Tektronix 5115) and on a ... recorder (ABEM 5651) writing intermittently with fast speed (10-200 cm/s). The amplifier was ... nected to a loudspeaker and to a discriminator equipped with a rate meter which could count either spikes exceeding a present value or only spikes with amplitudes between two determined levels. The ... of the rate meter, the arterial blood pressure and the mean left atrial pressure were recorded on a ... Grass polygraph (mod. 7). The ECG, the arterial and the left atrial pressure waves were also recorded ... the UV recorder together with the nerve activity.

Experimental procedure

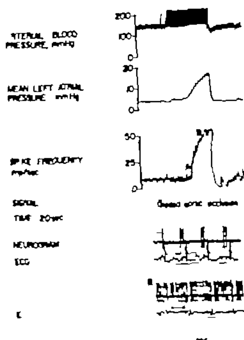
As a screening procedure to find receptors in the left side of the heart, the activity in all dissected filaments was observed during shortlasting (3-6 s) occlusions of ascending aorta. Every filament that responded increased activity to this manoeuvre was dissected further until a filament with only one active fibre was found. The receptors were localized by probing the heart with a thin probe in most instances after ... the animal and opening the heart. The total conduction time was obtained by placing stimulation electrodes connected to a Grass (S4) stimulator over the receptor site and recording the evoked potential in the filament in the neck. The conduction velocity was estimated from the approximated length of conduction pathway and the total conduction time.

Receptor activity was recorded during control conditions and during graded elevation of left ... pressure induced by graded aortic occlusion. The receptor response to electrically induced ventricular fibrillation of the ventricles was observed in some animals.

The significance of changes was determined by Students t test and values are presented as mean ...

Results

This study is based on recordings from 22 receptors in 15 normotensive rats. 4 of the receptors were dissected from the left cervical vagus and 18 receptors from the right cervical vagus.



Effects of progressive obstruction of the ... aorta on aortic blood pressure, mean ... pressure and spike frequency in ... left atrial C-fibre. The aortic blood pressure measured just above the aortic valve via ... in the right carotid artery. The letter ... spike frequency recordings corresponds to ... program below. Initially this receptor has ... cardiac modulated rhythm ... 1 2 imp/s. ... moderate increase in left atrial pressure ... (A) the receptor was firing ... 1 2 ... each wave. Upon further atrial distension there was a marked increase in receptor ... up to 16 impulses per cardiac cycle

total conduction time from the receptor site to the recording electrode was from 125 ms and the conduction velocities were from 0.4 to 1.2 m/s (mean ± S.E., 0.7 ± 0.1

Location

The receptors were located in the heart by probing with a fine plastic probe. Two right atrial receptors were located, one to the superior caval vein and the other to the base of the atrial appendix. The characteristics of the right atrial receptors were not studied in detail because the right atrial pressure was not measured. They however behaved similar to the left atrial endings with a clear cardiac rhythmicity upon activation during pulmonary artery occlusion. The left atrial receptors were located throughout the entire left atrium, in the pulmonary vein, left atrial junctions and in the left atrial appendix. 10 of the receptors were located in the opened non-beating heart and the other 12 in the intact heart. Of these 12 receptors 5 were located in the pulmonary veins close to the atrium and 4 was located in the appendix.

Response to increased atrial pressure

The receptors responded to increased pressure in the atria during a brief occlusion of the ascending aorta with maximal frequencies from 1 to 70 Hz (35 ± 4). The response of the receptors were also examined during ventricular fibrillation. Then there was a clear rise in atrial pressure and concomitant increased receptor firing as observed in left atrial C fibres in the cat (Thorén 1976).

Methods

Experiments were performed on 15 normotensive adult male Wistar rats ([illegible] weeks). In some animals the awake mean arterial pressure (MAP) was recorded via a PE [illegible] serted into the tail artery under light ether anesthesia. The animals were then anesthetized with pentobarbital (Nembutal® 40–50 mg/kg b w) i.a. or i.p. The anesthesia was maintained by [illegible] pentobarbital in repeated injections i.a. The trachea was cannulated with a PE 200 tube and pressure ventilation with pure oxygen was induced with a frequency of 28 per minute and end-expiratory pressure of 12 to 15 cm water In preliminary experiments (2 rats) this type of artificial ventilation [illegible] values for arterial PO (350–450 mmHg) and PCO (35–45 mmHg). To prevent acidosis [illegible] $NaHCO_3$ (0 1–0.2 mM) were injected at intervals. Muscle movements were prevented by gallamine (Flaxedil® 4–5 mg/kg b w) in repeated i.v injections. Rectal temperature was measured electronically [illegible] maintained at 37 38 C by means of a thermostatically controlled shielded heat pad.

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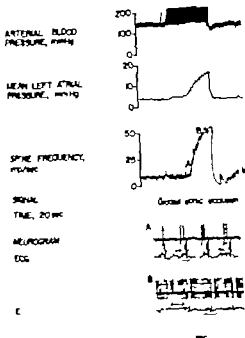
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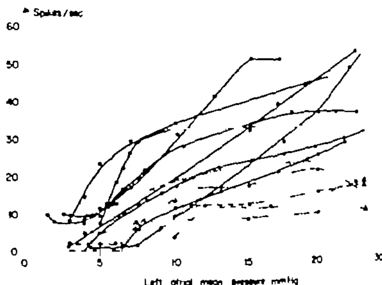
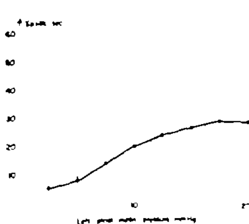


Fig. 2. The pressure-response curves for 13 different left atrial receptors in 12 rats. The interrupted lines indicate receptors of the low frequency type with maximal firing rates less than 25 Hz and the other receptors belong to the group of high frequency receptors. The sign ▲ indicates receptors with an irregular discharge pattern upon activation.

The response to a graded increase in left atrial pressure was also examined in 13 left atrial C fiber endings. The typical response is shown in Fig. 1 This receptor had a spontaneous activity of 1 or 2 impulses per cycle and after correction for the total conduction time (indicated by the asterisks) the receptor was activated during the v wave. Upon aortic occlusion there was a marked increase in left atrial pressure and a concomitant increase in firing with a maximal activity of 55 Hz or 16 impulses per cardiac cycle The thresholds for activation for all the left atrial receptors ranged from 2.5 to 5 mmHg in mean left atrial pressure



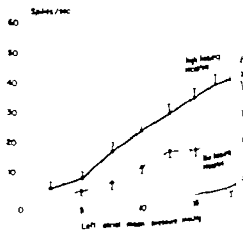
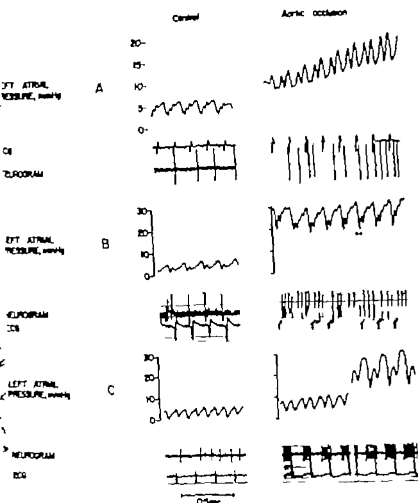


Fig. 3 A. Activity in non-medullated vagal afferents from the left atrium during increases in left atrial pressure caused by graded aortic occlusion (mean ± S E. of recordings from 13 single fibres).

Fig. 3 B Relationship between the mean left atrial pressure and firing frequency in 8 high frequency left atrial receptors and 5 low frequency left atrial receptors upon graded aortic occlusion (mean ± S E)



1 Left atrial pressure, ECG and activity in 3 left atrial C-fibre endings in 3 rats. The receptor in panel ... low frequent irregular discharge upon activation and is included in the low frequency type of re... The receptor in neurogram B is the only low-frequency receptor, showing cardiac rhythmicity ... elevation of left atrial pressure. The receptor in panel C is in the control situation activated alterna... in the a- and v-wave, but upon elevation of the left atrial pressure the receptor shows a progressive ... in relation to the v-wave. (The asterisks indicate the corrected position in the cardiac cycle of the ... activation.)

...verage, 5.4 0.5 mmHg). The pressure-response curves for the individual receptors are ...n in Fig. Some receptors showed very high firing frequencies up to 55 Hz and some ...ptors fired with rather low frequencies. Fig. 3 A shows the mean activity in this group ...eceptors in relation to left atrial pressure and is based on the data shown in Fig. 2.

...h and low frequency receptors

...surprising finding in this study was the marked variation in the pressure-response curves. ...the endings showed a regular discharge with very high maximal frequencies. These high

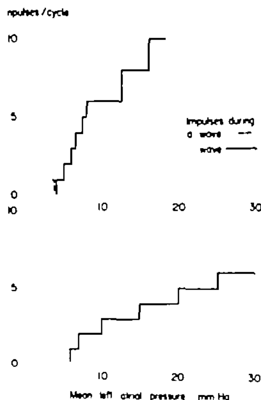


Fig. 5 Number of impulses per cardiac cycle in relation to the mean left atrial pressure for 2 left atrial C-f bre endings. Impulses generated during the a wave are plotted separately from those generated during the v-wave. At low pressure receptor show a low frequent a-wave discharge upon elevation of the left atrial pressure both receptors were markedly activated during the v-wave

frequency regular discharging receptors behaved very much as the medullated atrial receptors found in the cat (Paintal 1973) Other atrial C-fibre endings had similar characteristics to the earlier described atrial C fibres in the cat with a fairly low frequent activity. An attempt was therefore made to analyse the characteristics of high and low frequent receptors separately The receptors with maximal activity above 25 Hz were called high frequency receptors and receptors with maximal frequencies below 25 Hz were called low frequency receptors. In Fig. 2 the high frequency receptors are indicated by solid line and the low frequency receptors with an interrupted line. Fig 3 B shows the mean pressure-response curves for low and high frequency receptors separately

Is there then any evidence that these arbitory grouping of the atrial C-fibre receptors in rats really could represent 2 functionally different groups of receptors? Some evidence indicate that First the threshold for the high frequency and low frequency receptors was 4 6 ± 0 6 mmHg respectively 6 9 ± 0 7 mmHg (p < 0 05). Further all the high frequency receptors displayed a clear cardiac rhythmicity upon activation but 4 of the 5 low frequency receptors (as indicated in Fig. 2) displayed mainly irregular activity upon activation

The firing pattern in the cardiac cycle

The firing pattern in the cardiac cycle was established in 16 receptors because the conduction time from the receptor site to the recording electrode was measured. Upon activation 4 receptors showed an irregular discharge with no obvious relation to the cardiac cycle as indicated in Fig. 2. These receptors were of the low frequency type. All the other receptors showed a cardiac modulated rhythm upon activation with up to 16 impulses per cardiac cycle. The firing was mainly correlated to the v ... low pressure

had 1 3 impulses during each a-wave. Two receptors showed both v and a-wave rge, with 1 2 impulses per a-wave at high atrial pressures. The typical discharge in rent atrial C-fibre endings is showed in Fig. 4 Panel A shows a low frequency re and this ending displays irregular discharge during the control situation and during occlusion. Panel B shows the only low frequency receptor which displayed an evident c rhythmicity (indicated by the asterisks). Panel C shows a typical high frequency or This receptor shows in the control situation both an a- and v-wave discharge. the left atrial pressure is moderately increased the receptors discharged with 4–5 ses each v-wave and upon further increase in left atrial pressure the receptor discharge up to 10 impulses each -wave.

Fig 5 the number of impulses per cardiac cycle is plotted against the mean left atrial re The receptor in the top panel is at low pressures activated with 1 impulse each e. Upon pressure elevations the receptor discharge with up to 10 impulses per cardiac The receptor in the lower panel is only activated during the v-wave.

Discussion

ustence of atrial receptors with vagal medullated afferents has been known since many (*cf* Paintal 1973) These receptors have two different types of firing. One type of tors is discharging mainly during the -wave and these receptors are called B-receptors. ther type is discharging during the a-wa e and these receptors are called A-receptors. ver in the *in vitro* situation these two types of receptors have similar basic properties li *et al.* 1974) There is also a substantional population of endings with non-medullated afferents in the heart. The main receptor station in cats and dogs seems to be the left icle (Jarisch and Zotterman 1948, Coleridge *et al.* 1964 Sleight and Widdicombe 1965, én 1977) but there is also a lot of atrial C-fibre endings in the atria (Coleridge *et al* Thorén 1976). These atrial C-fibres in the cat respond mainly to increased atrial filling d some receptors also could respond to the atrial contraction (Thorén 1976). The holds for these receptors were somewhat higher and the firing frequencies were lower for the medullated receptors in the atria (Thorén 1976, Thorén *et al* 1976).

this study the characteristics of atrial receptors in the rat are described for the first time. rlier studies Knutsson and Sjöstrand (1974) have examined the characteristics of intra acic afferents dissected from branches of the superior laryngeal nerve in rats. However e authors never localized the receptors adequately and other studies indicate that the thoracic afferents in the superior laryngeal branches of the rat are not coming from heart but from the aortic arch (Andrew 1954)

ptor characteristics

in cats and dogs, atrial receptors i the rat respond mainly to atrial distension and are vated by low or moderate elevations in atrial pressure with thresholds from 2.5 to 9 Hg. Thus, a substantial population of the receptors are active at normal left atrial pres- es, hich is about 5 mmHg (Noresson *et al* 1978). Upon activation most of the receptors harge with a clear cardiac rhythmicity during the v-wave indicating atrial distension as main mechanism of receptor activation.

High and low frequency receptors

There is a marked variation in the receptor characteristics in the individual receptor receptors had low thresholds (2.5–5 mmHg) and respond upon atrial distension high firing rates up to 60 Hz and with cardiac rhythmicity Other receptors had higher thresholds (5–9 mmHg) and responded with low frequent (less than 25 Hz) discharge upon activation. The high frequency type of receptor seemed to ha properties to the medullated fibre group in the cat. The thresholds for the high freq receptor with cardiac rhythmicity were significant lower than for the receptors with frequent and irregular activity

An interesting question is then whether the high and low frequency receptors are in the same receptor system or constitute two different types of endings. As discussed some evidences are speaking in favour of the latter concept Thus, the low frequency receptors had significantly higher thresholds and discharged mainly (80) irregularly activation, indicating that they might constitute a different population of receptors. estingly enough, the aortic nerves of the rat also contains two types of afferent baroreceptor C-fibres. One type is discharging with high frequent regular activity and the other discharging with low frequencies and with a more irregular type of activity (Thorén 1977) It might thus be a general phenomenon in the rat that the afferent information baroreceptor and cardiac receptors is transmitted mainly in non-medullated fibres. reason might simply be that these animals are small enough to allow for a time delay afferent C-fibres. Interestingly enough the cervical vagus of the mice consists of 8 000 fibres of which the non-medullated fibres constitute 92 (Inoue 1975). This will also support the concept that the afferent information in small rodents is mainly mitted in C fibres.

Function of atrial receptors

In cats and dogs the function of the atrial medullated fibres seems to be different from function of the atrial C-fibres (Thorén *et al* 1976) It is thus of interest whether atrial frequency receptors in the rat have different function from the atrial low frequency receptors However recently the effect of elevation of left atrial pressure on the reflex inhibition renal sympathetic nerve traffic was examined in anaesthetized baroreceptor denervated (Ricksten *et al* 1978) Upon plasma volume expansion there was a marked inhibition renal nerve traffic due to activation of receptors in the left side of the heart and the threshold for the inhibition was about 4–5 mmHg which is in the same range as the threshold high frequency receptor in this study Thus, there is no evidence that the high freq C-fibres as recorded in this study should not induce a vasomotor inhibition in the same as the atrial C-fibres in the cat (Thorén *et al* 1976)

Absence of ventricular receptors in rats

Another peculiar finding in this study was that we were not able to localize any left ventricular receptors with non medullated afferents In the cat (Thorén 1977) and the dog ridge 1964 Sleight and Widdicombe 1965) the left ventricular C-fibre endings constitute large number of fibres and the left ventricle seems to be the major receptor station ferent C-fibres in the heart. There are at least two different exp tions why we

nd any left ventricular C-fibre endings in the rat. First, it is possible that the number ...re endings in the heart is so low or that the thresholds of these C-fibres are so high ...y for these reasons are difficult to find. Indeed, in two instances we recorded from ...located in the heart which was responding to aortic occlusion with a low frequent, ir ...discharge pattern and these receptors was markedly activated upon probing over ...tricular surface. However the definite localization of these receptors could not be ...ned in the opened nonbeating heart and thus the precise localization could not be ...ued. Another explanation is that the cutting of the vagal branches just above the ...ot might eliminate ventricular afferents but not the atrial afferents. However there ...ason to believe that atrial and ventricular afferents should ha e different afferent ...ys in the rat.

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Hepatic portal vein infusion of glucose on sodium excretion in the rat

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Abstract

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Anesthetized rats were prepared with catheters in the hepatic portal (HPV) and femoral (FEM) ve[illegible] the bladder or ureters. In some e periments the left kidney was denervated. HPV infusion of [illegible] at 2 ml/h for 20 ml increased Na excretion by the kidney when given as a second infusion. Bilateral vagotomy liminated the increase N excretion during glucose infusion and uncovered a delayed [illegible] in Na and K excretion. Renal denervation attenuated the increase in Na excretion to HPV glucose [illegible] infusion of glucose had variable effects, increas g Na excretion only after vagotomy. The re[illegible] interpreted to suggest that central and vagal receptors tend to enhance N excretion [illegible] nerve afferents and humoral mediator(s) have the opposite effect.

Key words glucoreceptors, hepatic portal vein, Na excretion, vagal afferent nerves, K excretion

Neurophysiological (Schmitt 1973 Niijima 1969) and behavioral (Blake and Lin [illegible] Novin, Sanderson and VanderWeele 1974) evidence exists for the presence of [illegible] sensitive receptors in the portal circulation to the liver The vagus apparently carr[illegible] hibitory information and the splanchnic nerves excitatory although both effects dep[illegible] experimental conditions including time of day (Schmitt 1973 Novin *et al* 1974) M[illegible] the work done has been related to food intake but hepatic portal infusion of 2 M [illegible] has been shown to stimulate saline drinking and suppress water drinking in rats (Blake [illegible] Lin 1978) This effect was not reproduced by femoral vein infusion of glucose or [illegible] portal vein infusion of equimolar sucrose or fructose (Blake and Lin 1978) there[illegible] ferentiating the system from that involved in suppressing food intake (Booth and [illegible] 1976)

We know of no studies on renal function during infusion of hypertonic glucose in[illegible] hepatic portal vein Hypotonic (2.5%) glucose had no effect on Na excretion different[illegible] femoral vein infusion (Daly Roe and Horrocks 1967) and similar negative results [illegible] reported for infusions of hypertonic sucrose (Passo Thornborough and Rothballer [illegible]

...of the above, we deemed it appropriate to examine the effect of hepatic portal ...n of hypertonic glucose on this other aspect of the salt and water balance system, viz ...retion of Na and water by the kidneys.

Methods

...ents were done on male Sprague-Dawley rats weighing between 250 and 350 g. All rats were ...ized with Inactin (Chem. Fabr. Promonta, GmbH, Hamburg, W. Germany), 120 mg/kg i.p. and ...ed at body temperature of about 37°C with servo-controlled heating pad. The trachea, one ...artery and both femoral veins were exposed and cannulated with polyethylene tubing. The ab... was opened by midline incision and, in all but two experiments, the appendix gently retracted to exposing mesenteric veins. One was catheterized through small needle hole and the tubing fixed by suturing to the mesentery. In two experiments only femoral vein infusions were made. In some ...bladder was cannulated directly for collection of urine, in others both ureters were cannulated ...urine from both kidneys collected in the same tube or if the left kidney was denervated, in separate ...Denervation was accomplished by stripping the pedicle of all visible nerve fibers (under dissecting ...ope) and applying crystals of phenol. (It was not crucial to the purpose of the experiment to assure ...e denervation by transplantation.)

...ial blood pressure was recorded from the femoral artery using Statham transducer and ink ...recorder. No blood samples were drawn. Urine volume was estimated by re-weighing of pre-weighed ...on tubes, analyses for Na and K concentration in urine samples were by atomic absorption spectro... Numerous urine samples were tested for the presence of glucose during periods of glucose infusion. ...rimental protocols were similar except for the order of glucose infusions, i.e. whether into hepatic ...or femoral vein first or second and whether or not vagotomy was performed. In the first 5 expts ...mals were given 3 to 5 ml of 0.85% NaCl solution i.v. over to 5 min and then saline was infused ...patic portal and femoral veins at 2 ml/h. In the remaining experiments, the rats received 1 ml of 5% ...ol in saline over 3 min period and were then given the mannitol solution into the femoral vein at ...and saline solution into the hepatic portal vein at 2 ml/h. One half (early experiments) to one hour ...the start of the infusions and completion of the operative preparation, the collection of 10 min urine ...was begun. After two to four "control" samples had been collected, 1 M glucose 2 ml/h was ...into femoral vein or the hepatic portal vein for 20 min. This infusion was added ... if infused ...hepatic portal vein, the saline being infused there was shifted to the second femoral vein. (In the ...periments with only femoral vein infusion, the 1 M glucose infusion was substituted for the 0.85 ...and was not additive.) After from three to five "recovery" periods (30 to 50 min) the 1 M glucose ...was repeated into the same or the other vein. In some experiments, as many as 4 glucose infusions ...were... In some experiments, bilateral cervical vagotomy (requiring 3 to 5 min) was carried out after two glucose infusions and then one or two more repeated. The numbers of infusions for each vein ...whether given as the first infusion of glucose or second (or more) are indicated with the results.

...ulation of percent change in urine flow, Na or K excretion was done by linear interpolation be... the immediately preceding control value and either the first or the third recovery period following ...infusion as described with the results. These approaches were used because a truly steady state was ...achieved; urine flow was sometimes increasing and sometimes decreasing. On ... number of occasions, ...an infusion was given just prior to peaking or troughing of an oscillation in excretion. Such infusions ...included because of the impossibility of using linear interpolation to predict values between. Statistical ...ences between means was by "t" test for unpaired values as well as by paired comparisons for inner... and denervated kidneys.

Results

...averaged values for urine flow, Na, and K excretion are shown for the studies on hepatic ...al vein (HPV) infusion of glucose in Fig. 1. Two control, two infusion, and three re...ery periods (all 10 min duration) are shown for each type of infusion. First infusion ...ns that glucose was infused into HPV before being given elsewhere and second infusion ...ns that glucose had already been given into either HPV or the femoral vein (FEM).

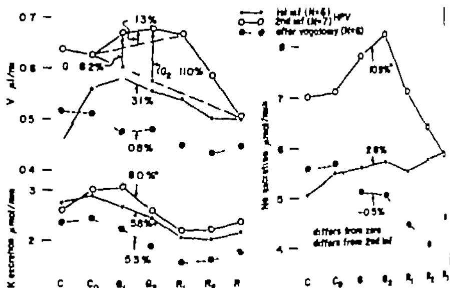
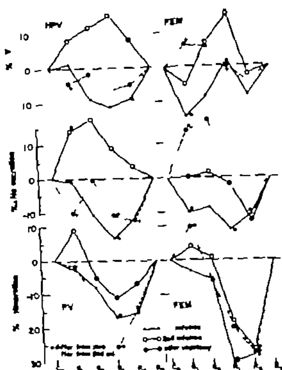


Fig. 1 Urine flow ($\dot{V}$), Na and K excretion before (C and Co), during (G and G_1), and after (R_1, R_2) HPV infusion of glucose For explanation of 1st and 2nd infusion and for calculation of percent ch see text

All but one of the post vagotomy infusions were second infusion or more. (There were differences between second or third infusions.) The two methods used to calculate per change in function are illustrated in the upper left part of Fig. 1 on the values for urine ($\dot{V}$) In the first, interpolation was between the control period just preceding glucose in (Co) and the first recovery period (R_1). The percent change was calculated from the differ between actual and predicted means for the average of G and G_2. Mean changes by method are included in Fig. 1 In the second approach, interpolation was between Co R and percent change from predicted was calculated for each of the intervening per namely G G_2, R and R_2. The latter approach is believed to be more acceptable Na and K excretion seemed to be altered following glucose infusion. For example, a de in K excretion following glucose infusion would seem more likely than the increase calculated by the Co to R method

The mean changes in urine flow Na and K excretion for each period during and glucose infusion are shown for early experiments in Fig. 2 and for late (unilateral den tion) experiments in Fig. 3 The effects of bilateral cervical vagotomy on the Na excr response to glucose infusion are given in Table I with the results from early experi combined with those from the late experiments, innervated kidney only The diffe in response between innervated and denervated kidneys is shown in Fig. 4 Occasional changes which differed from zero could occur by chance despite statistical signific Hence, only those findings which were reasonably consistent and usually found by methods of calculation will be considered to be demonstrated. These include primari increased excretion of Na seen during second infusion of glucose into HPV its re by vagotomy and attenuation by renal denervation

The pattern of response to HPV infusion of glucose was consistent for early an experiments (Fig. 2 and 3) First infusion (early exper) had no clear ch



HPV and FEM infusion of glucose ... cent change in urine flow Na and ... tion during and after the infusion ... as for HPV infusion same as Fig. 1 ... infusion were respectively 3, 4, ... for 1st, 2nd, and after vagotomy ...

... flow Na or K excretion. Second infusion of glucose increased Na excretion by the ... R method of calculation (Fig. 1) and during G by the Co to R approach (Fig. 2). ... to the point when all rats with innervated kidneys were considered, second infusion ... cose significantly increased Na excretion during and for 10 min after infusion (Table I). ... eral vagotomy eliminated the increase in Na excretion during glucose infusion and ... ed a significant decrease following infusion (Table I). The differences in responses ... e and after vagotomy were significant for all four periods, G G_a R and R_p. The ... r response of the denervated kidney is shown in Fig. 4 and applied to both Na and K ... tion. The N alue in the figure is the number of observations before and after vagotomy ... he results were equally significant if all comparisons on a given rat were averaged and ... as reduced to the number of rats.

... e pattern of response to FEM infusion of glucose was not the same for early and late ... riments. In the early experiments, first infusion of glucose decreased urine flow and ... excretion during the infusion and Na and K excretion afterwards (Fig. 2). In the lat ... riments, there was a trend for first infusion t increase water and electrolyte excretion ... ing the infusion which became significant in innervated kidneys after vagotomy (Fig. 3 ... Table I). Note that vagotomy had the opposite effect to that seen with HPV infusions. ... ral denervation may have depressed the increase in K excretion but had no effect on the ... excretion response (Fig. 3 and 4).

... the majority of experiments arterial blood pressure was unchanged by HPV or FEM ... sion of glucose. In two experiments, HPV glucose did increase blood pressure by 10 to

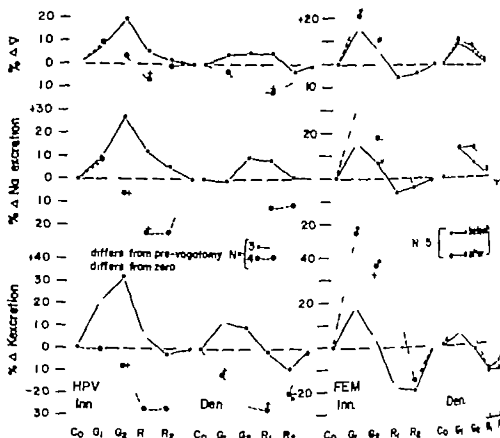


Fig. 3 HPV and FEM infusions f glucose on percent change in urine flow Na and K excretion and left kidneys followi g denervation of the left kidney The 3 pre-vagotomy HPV infusions were second infusions and the 5 pre-vagotomy FEM infusions were all first infusions.

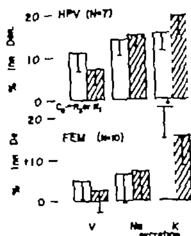
15 mmHg and slug injections of glucose into HPV (after urine collections had been pleted) almost invariably increased blood pressure as noted by Schmidt (1973) vagotomy increased blood pressure by 10 to 25 mmHg during the procedure and for 15 min thereafter Later pressure gradually fell and experiments were terminated if pressure fell below 95 mmHg.

Frequent testing of urine for glucose during infusion invariably gave negative results

TABLE I Percent change in Na excretion during and after infusion of glucose into HPV and FEM and after vagotomy

		(N)	G	G	R	R_2
				change mean ± S.E.		
HPV	before	(10)	12.5 ± 4.7^a	$20.1 \pm 7\ 1$	$9\ 7 \pm 4\ 2^a$	4.1 ± 4
	after	(9)	$-3\ 3 \pm 4\ 2^b$	$-3\ 1 \pm 2.3^b$	$-16.8 \pm 5.1^{a,b}$	$-17\ 3 \pm 4$
FEM	before	(9)	$8\ 6 \pm 5.9$	$4\ 3 \pm 2.2$	$-\ 4\ 3 \pm 3.6$	$-\ 7\ 7 \pm 8$
	after	(10)	$22.7 \pm 9\ 4^a$	$17\ 4 \pm 6.3^a$	$9\ 1 \pm 7.9$	$-\ 7.5 \pm 6$

[a] Differs from zero.
[b] Differs from pre-vagotomy



Percent change in function of the innervated kidney ...but of the denervated kidney (paired kidneys) calcu... the mean change during glucose infusion by both ... Co to R_1 and Co to R_2 interpolations. Values ...e flow, Na and K excretion are given for HPV and ...nfusions. A double asterisk indicates significance at ... probability level

Discussion

...ous studies in unanesthetized rats (Blake and Lin 1978) had shown that HPV infusion ...f glucose at 1 ml/h for 30 min increased saline and decreased H_2O intake. The purpose ... present studies was to determine if HPV infusion of glucose had an effect on Na excre... by the kidney that could be attributed to receptors located in the hepatic portal vein. ...ate of glucose infusion was the same as in the previous studies (1 M glucose at ml/h) ...hould not have increased plasma glucose concentration to more than 160 to 180 mg/ ...dl (Booth and Jarman 1976), well below the threshold for glycosuria. Consequently ...atriuresis could not be attributed to an osmotic diuresis from glucose excretion. The ...s were clear. Second infusion of glucose into HPV increased Na excretion prior to ...tomy but not afterwards. An additional observation was that renal denervated at... ted the response to HPV glucose during the time of infusion but not afterward. After ...tomy HPV infusion decreased Na and K excretion following the period of infusion. ...infusions of glucose tended to evoke a delayed decrease in Na and K excretion before ...omy but after agotomy Na and K excretion were increased during glucose infusion ...not significantly decreased afterwards.

...conclusion explanation of the results is not obvious. The following speculation is ...ed as the most parsimonious postulate but most certainly be considered to be over... ...ified and incomplete. There might be three receptors sensitive to glucose: central, ...chnic sympathetic afferents, and hepatic vagal afferents. There is good evidence for ...ree such receptors in the literature (Oomura *et al.* 1969, Schmitt 1973). FEM infusion ...d be expected to activate the central receptors more than the peripheral and HPV ...ion would activate the peripheral more than the central, especially first infusion of ...se. Second or more HPV infusion of glucose (the liver now saturated) would be ex... ...ed also to reach the central receptors. Both central and vagal receptors would tend to ...ate increased Na excretion, the latter by blocking neurally mediated decreases engendered ...thesia and operative procedures, see for example, Blake (1953). The splanchnic

glucose receptors would also tend to decrease Na excretion. Thus, FEM infusion, second would have little consistent effect, sometimes increasing sometimes depending on balance of central stimulatory and splanchnic inhibitory effects. HPV infusion would be similarly uncertain because of opposing vagal and splanchnic [illegible] HPV second infusion by adding stimulation of central receptors to that of vagal [illegible] would lead to increased Na excretion Vagotomy would eliminate the increase but [illegible] infusion but have little effect on the FEM infusion response. The tendency for [illegible] excretion to increase more after vagotomy with FEM infusion might be the result of [illegible] inhibition from other vagal afferents, *e.g* from cardiopulmonary receptors.

One difficulty with accepting central receptors as playing a role are the studies of [illegible] and Kaley (1976) They found that infusing 2.5 M glucose into the carotid artery of [illegible] had no significant effect on Na excretion However the dogs received sufficient [illegible] of vasopressin and 9α fluorohydrocortisone to eliminate effects mediated via these [illegible] mones. In conscious goats, infusion of hypertonic glucose into the carotid artery (or [illegible] valent) has a weak antidiuretic effect presumably due to release of some antidiuretic [illegible] (Eriksson Fernandez and Olson 1971) and Buckalew and Dimond (1976) have [illegible] that vasopressin is natriuretic by virtue of releasing a natriuretic hormone. This [illegible] might account for some of the increased Na excretion observed in the present studies [illegible] not for that fraction of the increase resulting from inhibition of neurally-mediated [illegible] in Na excretion Inhibition of the neurally-mediated decrease was seen only during [illegible] glucose infusion and not during FEM infusion. These observations, therefore, [illegible] to confirm a dual effect a central natriuretic effect and a vagally-mediated inhibition [illegible] sympathetically mediated decrease in Na excretion

The delayed decreases in Na and K excretion, pre-vagotomy with early FEM [illegible] and post vagotomy with HPV infusion, could not be attributed to enhanced renal [illegible] activity It is possible that these late decreases were the result of insulin secretion ([illegible] Lefebvre and Crabbé 1971) but we have no evidence to support such a conclusion.

In brief glucose infusion appeared to have conflicting effects on Na excretion [illegible] end result being dependent on variable activation of several different receptors. [illegible] vagotomy converted an increase in Na excretion during HPV glucose to a decrease [illegible] the infusion but had no effect on the variable response to FEM infusion.

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Lipoprotein-lipase activity of human skeletal-muscle and adipose tissue after intensive physical exercise

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Abstract

LITHELL, H K. HELLSING G LUNDQVIST and P MALMBERG *Lipoprotein-lipase activity of human skeletal-muscle and adipose tissue after intensive physical exercise* Acta physiol scand 1979 *105* 312–315

Seven young, healthy subjects performed bicycle exercise with a work g load leading to exhaustion after one hour of work The tests were done in the afternoon in the fed state. The serum insulin concentration decreased from 22 to 4 mU/l nd plasma glucagon increased from 41 to 340 pg l already after the ... work. The level of adipose tissue lipoprotein lipase activity (LPLA) did not fall as had been expected ... increased The skeletal muscle LPLA was uncha ged. The results indicate that during the first hour of heavy exercise the heparin-releasable LPLA n tissues is not influenced by the work induced changes ... serum hormone levels.

Lipoprotein-lipase activity (LPLA) is regarded to be the rate limiting factor in the transport of the fatty acid component of the serum triglycerides into tissues (Robinson 1970). In ... the effect of feeding has been thoroughly studied with regard to adipose tissue LPLA (Nilsson Ehle 1974 Pykälistö Smith and Brunzell 1975 Nilsson-Ehle, Carlström and Belfrage 1975 Lithell *et al* 1978 a) but less so with regard to skeletal-muscle LPLA. However recently we reported that the LPLA of skeletal muscle tissue was lower in the fed than in the fasting state during an ordinary day of work (Lithell *et al* 1978 a)

It has been shown in man that the increase of LPLA of adipose tissue in the fed state is dependent on the increment of serum insulin above basal level (Pykälistö *et al* 1975, Nilsson-Ehle *et al* 1975). In contrast to this, the skeletal-muscle LPLA has been reported to be inversely related to the fasting serum insulin concentrations (Lithell *et al* 1978 b). Skeletal muscle LPLA in the rat increased after glucagon perfusion *in vitro* (Borensztajn, Keig and Rubenstein 1973) Studies in man have shown that when the ratio between insulin and glucagon is relatively low as in the fasting state (Lithell *et al* 1978 a) or during clofibrate therapy (Lithell *et al* 1978 c) skeletal muscle LPLA is increased.

In fed rats exercise leading to exhaustion after 1 h decreased the LPLA of adipose tissue

[increased LPLA of heart-muscle tissue (Nikkilä, Torsti and Penttilä 1963). As serum [insulin] decreases and glucagon increases during exercise (Bottger, Faloona and Unger 1971), [c]hanges in tissue LPLA may have occurred due to the changed levels of circulating [horm]ones.

[The] present study was undertaken to find out whether tissue LPLA in man changed in a [simila]r manner after heavy physical work for 1 h.

Materials and Methods

[Subjec]ts

[Seven h]ealthy subjects (1 female, 6 males) with mean age of 24 years (range 22–28 years) declared themselves [willin]g and consented to take part in the study.

[Experi]mental schedule

[The st]udy started at 2 p.m. and the subjects had been doing their ordinary physically light work during [the ear]ly part of the day and had eaten breakfast, lunch and two small meals. Specimens of subcutaneous [adipos]e tissue of the abdomen were taken by needle-biopsy technique (Hirsch and Goldrick 1964) and of skeletal muscle tissue of the m. vastus lateralis as earlier described (Bergström 1962). Blood samples [were] taken for determination of insulin and glucagon.

[Aft]er the first biopsy the subjects rested for 1 h and then performed ergometer bicycle exercise for 1 h. [The w]orking load was chosen so that the subjects were totally exhausted after 1 h of exercise (see Methods [of an]alysis).

[Aft]er 30 and 60 min of work blood samples were taken for glucagon and insulin determination. Tissue [specim]ens were again taken immediately after the exercise.

[Meth]ods

[Tissue] specimens were stored frozen in liquid nitrogen and the LPLA of the tissue specimens from one [subje]ct were later analysed on the same experimental day. The tissue specimens were incubated at 37°C [in a] medium containing heparin (1 g/l) and the LPLA was determined as the release of fatty acids [from] serum-activated Intralipid emulsion prepared by Vitrum AB, Sweden, using triolein-triolein as [substrat]e (Lithell and Boberg 1977; Lithell and Boberg 1978). The enzyme activity was expressed as the [amoun]t of fatty acids (FA) per gram tissue and minute (mmol FA g^{-1} min^{-1}).

[Plas]ma immunoreactive insulin was analysed in quadruplicates using the Phadebas Insulin Test (Pharmacia Diagnostics AB, Uppsala, Sweden) based upon the radioimmunosorbent technique described by [Wide], Axén and Porath (1967). The insulin concentration is expressed as milli-units/litre (mU/l). Plasma [immuno]reactive glucagon was analysed according to Unger *et al.* (1970), using glucagon antiserum 30 K, [whic]h is specific for pancreatic glucagon (Aguilar Parada, Eisentraut and Unger 1969). The blood samples [for g]lucagon analyses were collected in chilled tubes containing heparin and the protease inhibitor Trasylol [(Baye]r, Leverkusen, Germany; 400 KIE/ml of blood). The specimens were immediately centrifuged and [the] plasma was frozen and stored at –70 C until assayed. Pork glucagon (Novo, Copenhagen, Denmark) [was] used as standard. Duplicate analyses were made. The maximum work load that could be sustained for [6 min] (W max) was estimated in each subject on electrically braked ergometer bicycles, beginning at a work [load] of 100 W and increasing by 100 W every 6 min until exhaustion. W max values ranged between 165 [and] 320 W in the subjects. The W max value was used to predict the work load that could barely be tolerated [for] 60 min (W 60) using the formula K = (log 6 – log 60)/(log W max – log W 60), where K was chosen [betw]een 5 and 6 according to the degree of physical fitness of the subject (Tornvall 1963). The W 60 thus [calcul]ated varied between 63 and 68 % of W max. The one-hour exercise was performed on mechanically [brake]d ergometer bicycles. The work load was adjusted guided by the heart rate at 6 min and slight further [adjust]ments were performed during the run in some of the subjects as guided by heart rate and appearance [of t]he subject. At 60 min all subjects were completely exhausted with heart rates above 190.

[St]atistics

[Dif]ferences between results before, during and after work were tested for statistical significance using [pair]ed t-test (two-tailed analysis) (Snedecor and Cochran 1971).

TABLE I The lipoprotein lipase activity (LPLA) of adipose and skeletal-muscle tissue before and after exercise for 1 h, the serum insulin and plasma glucagon concentrations before and after 30 and 60 min of exercise (mean ± S.D.) in 7 healthy subjects are given. Paired t-test was used in calculation of the mean change between zero time and 30 and 60 min resp. and the degree of significance is indicated (* = 2p < 0.05 ** = 2p < 0.01).

	Adipose-tissue LPLA nmol FA g⁻¹ min⁻¹	Skeletal-muscle LPLA nmol FA g⁻¹ min⁻¹	Serum insulin mU/l	Plasma glucagon pg/l
Before work (mean ± S.D.)	97.4 ± 25.7	27.2 ± 8.2	21.7 ± 15.3	241 ± 1[illegible]
After 30 min work (mean ± S.D.)	—	—	4. ± 2.4*	340 ± 2[illegible]*
After 60 min work (mean ± S.D.)	140.3 ± 45.0	23.0 ± 8.5	8 ± 1.5**	377 [illegible]

Results

The LPLA of adipose tissue increased significantly from 97 to 140 nmol FA g⁻¹ min⁻¹ (Table I). The mean LPLA of skeletal-muscle tissue was 27 (nmol FA g⁻¹ min⁻¹) before and 23 after the physical work ($0.05 < p < 0.10$) (Table I).

The mean serum insulin concentration being 21.7 mU/l before the physical work decreased to 4.2 mU/l after 30 min of work and was 2.8 mU/l at the end of the work (Table I).

The glucagon concentrations were on the average 241 pg/l before the work started and increased to 340 after 30 and were 377 pg/l at the end of the work (Table I).

Discussion

The present study showed that in non-fasting subjects performing heavy physical exercise for 1 h the LPLA of adipose tissue increased but not that of muscle tissue. The insulin concentration dropped dramatically during the exercise test but in spite of this the adipose-tissue LPLA increased. In the rat, adipose-tissue LPLA decreased during an equally long [illegible] (Nikkilä, Torsti and Penttilä 1963). This increase in man may be a delayed effect due to the high insulin concentrations during the 6 h of fed condition preceding the experiment. In fact, Pykälistö *et al.* (1975) did not find a significant increase of adipose-tissue LPLA in nutritional experiments until 6 h had passed after breaking the fast. The fall of insulin in the present study to low levels may have been too short to affect the time-dependent [illegible] of heparin-releasable LPLA in adipose tissue.

In the skeletal muscle the LPLA did not change. In the rat, only the LPLA of heart muscle increased after exercise, but not that of skeletal muscle (Nikkilä *et al.* 1963, Askew *et al.* 1972). The ratio between insulin and glucagon concentrations after heavy work should favour an increase of LPLA in skeletal muscle according to the results of earlier studies (Lithell *et al.* 1978 a, Lithell *et al.* 1978 c). It is possible that the LPLA in muscle tissue responds during exercise to other factors than insulin and glucagon. *E.g.* the muscle contractions may lead to local changes within the fibres of the working muscle causing an increase of muscle LPLA. The present study shows that bicycle exercise leading to exhaustion after 1 h of work does not result in an increase of LPLA of the lateral vastus muscle. [illegible]

preliminary data obtained after several hours of exercise have demonstrated a marked [illegible]se in skeletal-muscle LPLA (to be published). It thus seems as if the synthesis of the [illegible]n-releasable muscle LPLA is time-dependent as is that of adipose-tissue LPLA, [illegible]er caused by hormonal changes or local environmental factors.

[illegible]dy was financially supported by the Swedish Medical Association and the Swedish Medical Research [illegible] (B79-19X-05446-01).

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Axonal transport of adrenaline, noradrenaline and phenylethanolamine-N methyl transferase (PNMT) in sympathetic neurons of the cod, *Gadus morhua*

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Abstract

ABRAHAMSSON T *Axonal transport of adrenaline noradrenaline and phenylethanolamine-N-methyl transferase (PNMT) in sympathetic neurons of the cod, Gadus morhua* Acta physiol. scand 1979 *105* 316–325

The axonal transport of adrenaline noradrenaline nd phenyletha olamine-N-methyl transferase (PNMT) has been studied in ivo I sympathetic neurons of the splanchnic nerve in the cod, *Gadus morhua*. Adren aline and noradrenaline are transported at mean a onal transport rate of 16 mm/day. After corr for non-mobile fraction of adrenal ne and noradrenaline which does not contribute to the amine acc lation proximal t a ligature a maximal rat of transport was calculated to bout 45 mm/day for b amines. The ncreased level of catecholamines in front of a ligature could be depleted by reserpine ment, which trongly suggest that both amines are stored gra ules. PNMT is transported at a of 2 mm/day The subcellula distribution of the cod PNMT was exclusively non-particulate. Th PNMT was further characterized by studying substrate pecificity temperature and pH optima. It cluded that adrenaline and noradrenaline, stored in gra ules, are transported i a proximo-distal d t a high rate compared to PNMT

Key words. Axonal transport catecholamines, PNMT *Gadus morhua*, teleost fish, sympathetic system

In mammalian sympathetic neurons, axonal transport of noradrenaline and enzymes volved in the synthesis of catecholamines, has been studied by numerous investigators has been shown that granular vesicles containing noradrenaline are transported in a prox distal direction at a high rate of 5–10 mm/h (Dahlström and Häggendal 1966, Banks 1969 Häggendal *et al* 1975) This transport rate is similar to that of other granule-b substances such as dopamine β-hydroxylase (EC 1 14 17 1) (Wooten and Coyle 1 Brimijoin 1975) and chromogranin A (Geffen *et al* 1969) Non-granular enzymes su DOPA-decarboxylase (EC 4 1 1.26) are transported in a proximo-distal direction at a rate of 0 6–3 0 mm/h (Oesch *et al* 1973 Wooten and Coyle 1973). It has been sugg (Oesch *et al* 1973) that the rates of axonal transport for the enzymes involved in the synthesis of noradrenaline are well correlated to their subcellular distribution. How

...n and Coyle (1973) failed to confirm the suggestion that particulate enzymes are ...orted rapidly and soluble enzymes slowly and Brimijoin and Wiermaa (1977), studying ...onal transport of tyrosine hydroxylase (EC 1 14.3.a), found no evidence that the ...y transported fraction had a different subcellular distribution from that of the slower ...orted or stationary fraction.

...mammals adrenaline and also phenylethanolamine-N-methyl transferase (EC 2.1 1 10) ...IT), which is the enzyme catalyzing the conversion of noradrenaline to adrenaline, are ...y found in the adrenal medulla (Kirshner and Goodall 1957 Axelrod 1962) but small ...ounts hav also been reported from different regions of the mammalian brain (Hok... *al* 1974, Saavedra *et al.* 1974). In amphibians, PNMT and adrenaline have been ...strated in many tissues (Euler 1963, Falck *et al.* 1963 Angelakos *et al.* 1965 Wurt... *et al.* 1968) and Wooten and Saavedra (1974) showed that PNMT is transported at a ...low rate of axonal transport in the toad, *Bufo marinus*, sciatic nerve. The subcellular ...ution of PNMT in mammalian adrenal medulla is mainly non-granular (Axelrod ...hich was also the distribution observed in the toad sciatic nerve (Wooten and ...dra 1974)

...many teleost fish, adrenaline is present in larger quantities than noradrenaline in blood ...a and in the majority of tissues (Euler and Fänge 1961 North 1965 *cit in* Burnstock ... Gannon and Burnstock 1969 Mazeaud 1971 Abrahamsson and Nilsson 1976). In ...od, *Gadus morhua*, the presence of PNMT has been demonstrated in sympathetic ...ns, indicating the possibility that in different tissues adrenaline can be synthetized ...y from noradrenaline (Abrahamsson and Nilsson 1976). It has also been shown that ...adrenaline and noradrenaline are stored in post-ganglionic sympathetic nerve endings ...hus both may act as transmitter substances (Abrahamsson and Nilsson 1975).

...further describe the organization and function of adrenergic neurons in teleost fish, ...dy was performed to determine the characteristics of adrenaline, noradrenaline and ...IT axonal transport in the sympathetic neurons of the splanchnic nerve of the cod, ...morhua.

Materials and Methods

...he cod, *Gadus morhua*, of 800– 000 g b wt. were kept at 10°C in circulating ...all-aerated sea ...ater ...g operation the animals were anaesthetized with MS 222 (tricaine methane sulphonate, Sandoz) ca ...1 sea water The splanchnic nerve, ...hich in teleost fish consists mainly of post-ganglionic sym... ...ic fibres from the celiac ganglion, was ligated (silk suture 6–0) through an incision on the right side of ...ody After suturing the incision, the fish were kept in aquaria at 10°C for different periods before they ...killed and segments of the splanchnic nerve were dissected out and used for measurements. The ...mal 1 cm of the nerve just above the ligature was taken for the accumulation study In some groups the ...just distal to the ligature or the 1 cm ...hich begins 5 mm distal to the ligature were also dissected out ...[illegible] In one group of experiments, reserpine (Serpasil CIBA) 2.5 mg/kg b wt. was given ...in ...and 30 h after the ligation of the splanchnic nerve. The fish were kept in aquaria at 10°C and killed ...after the operation The 1 cm nerve segment proximal to the ligature was removed for catecholamine ... The doses of reserpine used have previously been shown to deplete the catecholamines in the cod ...s (Abrahamsson and Nilsson 1975)

...metric determination of ...atecholamines

...e segments ...ere homogenized in 5 ml 0 4 m ice-cold perchloric acid containing 200 µl 10% EDTA and ...µl ...ascorbic acid. The nerve segments were analyzed for their content of adrenaline and noradren-

aline by the fluorimetric method of Bertler *et al.* (1958) as modified by Häggendal (1963), including purification of tissue extracts on Dowex 50 W X8 resin. The recovery of noradrenaline (100 ng) and ... (100 ng) from the columns was determined and found to be: noradrenaline 83 ± 2.1 (n = 11) ... 8 ± 1.6 (n = 11). The results given below are corrected for these recovery values.

Phenylethanolamine-N-methyl transferase (PNMT) activity

PNMT was assayed by the method of Axelrod (1962). Nerve segments were homogenized in ... 0.14 M KCl, and 100 μl of the homogenate was incubated at 30°C for 30 min in a total volume of ... containing 0.01 M phosphate buffer pH 7.9, 0.4 mM normetanephrine and 2 nmol of ^{14}C-labelled methionine (specific activity 56 mCi/mmole, Amersham Radiochemical Centre). A control in which the substrate (normetanephrine) was omitted was run together with each sample.

The subcellular distribution of PNMT was studied in the celiac ganglion and the splanchnic nerve ... tissue was homogenized in 2 ml ice-cold 0.25 M sucrose and the homogenate was centrifuged at ... for 15 min, the resulting supernatant was centrifuged at 100 000 *g* for 60 min to get a "microsomal" ...

To study the substrate specificity for cod PNMT, homogenates of the splanchnic nerve were ... with a variety of amines at a concentration of 0.4 mM. The ^{14}C-methyl labelled products were ... into organic solvents as described by Axelrod (196) and Saavedra *et al.* (1973).

The activity of PNMT is expressed as U/g or mU/cm where one unit (U) equals 1 nmol of ... product formed from the substrate per h.

Fluorescence histochemistry

Segments from the splanchnic nerve were dissected out and quick-frozen, freeze-dried and treated with formaldehyde vapour for 3 h at 80°C as described by Falck and Owman (1965). Sections of 10 μm ... mounted in Entellan (Merck) and viewed in a Leitz Ortholux microscope equipped for fluorescence microscopy.

Statistics

The method of least square were used to calculate regression curves and the Student's t-test for ... tion of the significance of difference between means (Bailey 1958). Values are expressed as mean ± SE...

Results

Temperature and pH optima for PNMT are shown in Fig. 1. The substrate specificity ... cod PNMT is summarized in Table I and shows that phenylethanolamine and normetaneph... rine were substrates for cod PNMT while phenylethylamine was not. The distribution ... for PNMT in various subcellular fractions show that the PNMT activity was ...

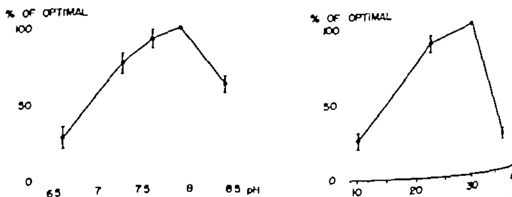


Fig. 1. Effects of pH and temperature on the activity of cod PNMT in the celiac ganglion/splanchnic ... The enzyme activity is expressed as per cent of optimal activity in each series of expts. Number of ... tions at each point = 6.

[illegible] I. The substrate specificity for cod N-methyl transferase. The results are mean values expressed as % of activity obtained with phenylethanolamine as substrate. Number of experiments = 4

[illegible]	N-methyl transferase activity
[illegible]	100
[illegible]	1
[illegible]	9
[illegible]	79
[illegible]	1

in the non-particulate fraction both in ganglion celiacum and splanchnic nerve (Table II).

[illegible] anatomy of the splanchnic nerve trunk is very different from one animal to another [illegible] with side branches and sometimes the nerve splits up into 2 or 3 parallel trunks which [illegible] parately (Stannius 1849, Nilsson 1972). A histochemical study of the cod splanchnic [illegible] (outside the celiac ganglion) revealed the presence of a very small amount of fluo[illegible] ganglionic cells which occasionally could be seen in small clusters (Fig. 2 a).

[illegible] cod splanchnic nerve was ligated for periods of 1–6 days. The fluorescent histo[illegible] ical study showed large amounts of strongly fluorescent material proximal to the [illegible] re after 3 days (Fig. 2 b) indicating that catecholamines are transported in a proximo-[illegible] direction. The accumulation of adrenaline and noradrenaline proximal to a ligature [illegible] rapid and linear for the first 3 days (Fig. 3). Reserpine, given at a time when a certain [illegible] nt of adrenaline and noradrenaline already have accumulated above the ligature, [illegible] d a highly significant reduction of catecholamine content proximal the ligature, [illegible] ured 3 days after ligation (Fig. 4). PNMT which was also found to accumulate above [illegible] ture, shows a quite different pattern, with a slow and linear accumulation during the [illegible] 6 day period (Fig. 5). Regression curves were calculated for the linear portion of the [illegible] mulation proximal to the ligature and from the slope of each regression line a mean [illegible] of axonal transport was estimated: adrenaline 16.5 mm/day, noradrenaline 15.9 mm/[illegible] and PNMT [illegible] mm/day. To estimate the absolute rates of transport for the rapidly ac[illegible] mulating substances, the proportion of adrenaline and noradrenaline free to move distal [illegible] ligature was calculated. The catecholamine content in the 1 cm segment which begins [illegible] m distal to the ligature was measured the 1st and 3rd day after ligation. The results

[illegible] II. Subcellular distribution of cod PNMT in celiac ganglion and splanchnic nerve. The results are means [illegible] values expressed as U/g tissue. Number of experiments = 3.

[illegible] fraction	PNMT activity	
	Celiac ganglion	Splanchnic nerve
[illegible] g sediment	0.1±0.1	0.1±0.1
[illegible] g sediment	0.2±0.1	0.1±0.1
[illegible] g supernatant	16.3±0.9	13.7±2.5



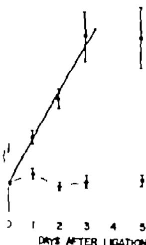
Fig. — Fluorescent histochemical microphotographs showing (A) the presence of fluorescent [illegible] cells in the splanchnic nerve (outside the celiac ganglion) and (B) the accumulation of catech[illegible] proximal to a ligature (arrow) of the splanchnic nerve Calibration in A = 50 µm and B 100 µm

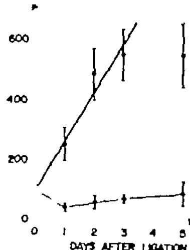
(Fig. 6) show that the stationary portion for both adrenaline and noradrenaline is appr[illegible] mately 60–65 The estimated absolute rates of catecholamine transport would then be adrenaline 46 mm/day and noradrenaline 42 mm/day at a temperature of 10°C.

Discussion

The disappearance of only a fraction of adrenaline and noradrenaline distal to a lig[illegible] shows the presence of a non-mobile fraction which does not contribute to the catechol[illegible] accumulation above a ligature. Thus, in order to calculate the true rapid axonal tra[illegible] rate, correction for the non mobile fraction must be considered (Häggendal *et al* 19[illegible] After this correction it was found that both adrenaline and noradrenaline are transp[illegible] in a proximo-distal direction at a rate in the order of 45 mm/day The depletion of [illegible] amines above the ligature after reserpine treatment strongly indicate that both amine[illegible] preferentially stored in granules. The observations suggest that the observed axonal t[illegible] port of catecholamines in cod sympathetic neurons correspond to the fast axonal tra[illegible] of noradrenaline storage granules in mammalian sympathetic neurons (Dahlström [illegible] Häggendal 1966, Banks and Mayor 1972)

Campbell and Gannon (1976) reported the presence of fluorescent ganglionic ce[illegible] regularly clustered along the splanchnic nerve of *Salmo trutta* and *S gairdneri* A very

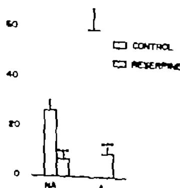




...ne levels in the splanchnic nerve 1 cm proximal to (●—●) and 1 cm distal to (●—●) The adrenaline and noradrenaline content in 1 cm untreated nerve segment at zero time is, adrenaline: 9.6±1.5 ng (100±14.5 %) (n 13) and noradrenaline: 5.0±0.7 g (100±14 %) (n 13). All values ...ssed as per cent of these control values. Number of experiments at each point = 9–13. Equations ...near phase of accumulation proximal to the ligature were, adrenaline: Y = 95±165 X (= 45) ...oradrenaline: Y 110 159 X (n 45). Correlation coefficients (adrenaline: = 0.75, noradrenaline ...7) were significant at p 0.001 for each regression line. Number of expts. = n.

...nt of fluorescent ganglionic cells were also found in the cod splanchnic nerve, these ...might store catecholamines and PNMT and it cannot be excluded from this study ...hese cells to some extent interfere with the nerve segment measurements.

...MT is transported at a much slower rate than the catecholamines. This rate of flow is ...ar to that previously reported for PNMT transport in amphibian sciatic nerve (Wooten ...Ben-Ishay 1974). The subcellular distribution of cod PNMT in the splanchnic nerve was



...4. Histogram showing the effect of reserpine on the ...aline and noradrenaline level accumulating in the 1 cm ... segment proximal to ligature of the splanchnic ...n. The time for ligation at 3 days. Number of expts. ... (controls) and 8 (reserpine). Levels of significance: ...p 0.001

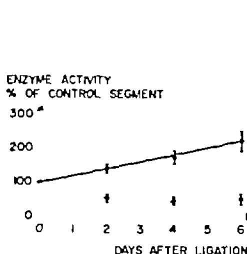


Fig. 5

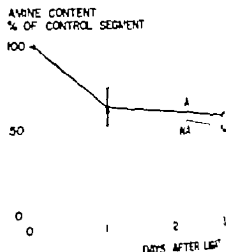


Fig. 6

Fig. 5 PNMT activity in the splanchnic nerve 1 cm proximal to (● – ●) and 1 cm distal to (● – ●) the ligature. In 1 cm untreated nerve segment at zero time the activity of PNMT was 5.3 ± 0.6 mU (n = 9). The values are expressed as per cent of this control value. Number of expts at each period ... Equation for the accumulation period proximal to the ligature was: Y = 99 + 20 X (n = 18). Correlation coefficient (r = 0.59) was significant at $p < 0.001$ for the regression line. Number of expts ...

Fig. 6. Adrenaline and noradrenaline content in the 1 cm nerve segment which began 0.5 cm ... ligature of the splanchnic nerve. All values are expressed as per cent of the content in 1 cm untreated ... at zero time. For absolute values of controls see legend to Fig. 3. Number of expts at each period ... Levels of significance: $p < 0.05$, $p < 0.01$, $p < 0.001$

exclusively non particular which is in agreement with the hypothesis that non-particulate enzymes are transported at a very slow rate (Oesch *et al.* 1973). However it is not evident from this study that a small fraction of PNMT is transported at a much higher rate. To be able to elucidate the problem whether PNMT axonal transport can be separated into different fractions a more accurate method ought to be used (Edström and Hanson ...; Brimijoin 1975).

In mammalian peripheral nerve fibers the velocity of rapid axonal transport is ... 400 mm/day (Ochs 1972, Ochs and Jersild 1974) which is very much faster than the observed fast rate in this study. McLean and Burnstock (1972) reported that in toad sympathetic neurons the accumulation of adrenaline fluorescence in front of a ligature occur at a ... rate compared to noradrenaline accumulation in rat sciatic nerve (Dahlström 19..). In general the fast axonal transport rate in the nervous system of poikilothermic vertebrates are slower compared to mammalian neurons (*Grafstein et al.* 1972, Edström and Hanson 1973). Since the fast axonal transport phase is temperature dependent (Edström and Hanson 1973) the difference might partly be explained by the circumstances that the experiments on poikilothermic animals are carried out at a temperature below 37 °C corresponding to the optimal temperature for the species examined. In goldfish optic nerve the fast transport rates observed at 20.5 °C and 9 °C were about 60 mm/day and 70 mm/day respectively (Grafstein *et al.* 1972), the rate at 9 °C is similar to the observed fast axonal transport at 10 °C in cod sympathetic neurons.

results of the present study provide further evidence for the occurrence of PNMT in thetic neurons of the cod and support the previously presented hypothesis that line can be synthetized in sympathetic neurons and act as neurotransmitter (Abrahamsson and Nilsson 1975, 1976). In a preliminary study the pH and temperature optima of PNMT have previously been reported (Abrahamsson and Nilsson 1976). In this however it was shown that the temperature optimum is below that previously reported (37°C). The temperature optimum of 30°C for cod PNMT is similar to the observed um for cod dopamine-β-hydroxylase (Jönsson and Nilsson 1978).

substrate specificity of cod PNMT in sympathetic neurons for β-hydroxylated phenylethanolamines such as phenylethanolamine and normetanephrine closely correspond to substrate specificity of PNMT obtained from mammalian adrenal medulla and amphibian nerve and adrenal gland (Axelrod 1962, Wurtman *et al.* 1968, Wooten and Saavedra 1974). Thus the activity of the N-methylating enzyme measured in cod splanchnic is not due to a non-specific N-methyltransferase of the type previously described in and toad brain (Saavedra *et al.* 1973).

e results from this study confirm the earlier observation that both adrenaline and noradrenaline are stored in an intragranular compartment (Abrahamsson and Nilsson). Since this study strongly indicates that PNMT is located in an extragranular compartment, noradrenaline must come out of the granules to be N-methylated to adrenaline h, afterwards, returns to the granules to be stored. This would be similar to the presented thesis pathway for adrenaline from noradrenaline in mammalian adrenal medulla (Axelrod 1962). However Laduron (1972) argued against this translocation mechanism for adrenaline synthesis, he proposed an alternative pathway for adrenaline synthesis *via* amine and epinine which is taking place inside the granules. Results against this hypothesis presented by Pendleton *et al.* (1976) showed that the noradrenaline level in the mammalian adrenal medulla was increased after PNMT inhibition, indicating that noradrenaline precursor for adrenaline. Whether noradrenaline or any other amine is the precursor of adrenaline in the cod adrenergic neurons is still to be clarified.

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The effect of water temperature on the hormonal response to prolonged swimming

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Abstract

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The relationship between thermoreception, hormonal secretion and muscular activity was studied in ... swam 60 min in 21 27 and 33 C wate at a speed requiri g 68 % of V_{O_2} max (determined in 27 C ... Rectal temperature increased in 33°C (1.3 ± 0.. °C, mean nd S.E.) and 27°C (0.7 ± 0.1°C) expts ... decreased in 21°C expts. (0.8 ± 0.3 C) Changes in esophageal and muscle temperatures paralleled ... in rectal temperature. Plasma noradrenaline was higher i 33 C than in 27°C expts. and growth hormone ... cortisol and glucagon concentrations increased in 27°C and 33°C expts. only Insulin concentrations were uniformly depressed during swimming at the different water temperatures. In 21°C expts. noradrenaline and drenaline concentrations were higher than in 27°C expts. V_{O_2}, carbohydrate combustion and ... lactate were slightly lower i 33 C expts. Plasma glucose decreased slightly and FFA and glycerol concentrations increased identically n all expts. Heart rate increased continuously during swimming in 27 C ... 33°C expts., but ot in 21°C expts. In conclusion the rise in body temperatures normally observed during exercise enhances the exercise nduced increases in the plasma concentrations of noradrenaline, ... growth hormone and glucagon Decreased body temperatures may elicit catecholamine secretion ... direct consequence of thermoreception Shivering may ccount for previously observed decrease in ... secretion during cold stress but not for ncreases in cortisol nd growth hormone.

Key words. Insulin, glucagon, cortisol, growth hormone, epinephrine norepinephrine, exercise, ... heat, cold

During prolonged exercise in a neutral thermal environment a progressive, time dependent tachypneic hyperventilation and hypocapnia and a continuous rise in heart rate ... splanchnic vasoconstriction take place This ventilatory (Dempsey *et al* 1975) and cardiovascular (Rowell 1974) "drift is accompanied by and probably—at least in part—mediated by gradually rising body temperatures. Also the hormonal response to heavy ...

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...ise an increase in the concentrations in plasma of catecholamines, glucagon, growth ...ne and cortisol and a decrease in insulin concentrations—is accentuated with the ...tion of exercise (Galbo *et al.* 1979). Since the described hormonal changes are similar to ...reported often to be associated with heat stress (Collins and Weiner 1968, Maher *et al.* ...Gale 1973, Rayfield *et al.* 1973, Rocha *et al.* 1973, George *et al.* 1974, Francella *et al.* ...the possibility exists that the exercise induced increase in body temperature enhances ...ormonal response to exercise, too.

...hormonal response to cold is generally considered as a direct consequence of ...rception. However also this response bears resemblance to the hormonal response ...rcise (Baum and Porte 1971, Chin *et al.* 1973, Gale 1973, Östman and Sjöstrand ...Tedesco *et al.* 1976, Forichon *et al.* 1977, Johnson *et al.* 1977, Usategui *et al.* 1977). ...cold exposure may elicit shivering the hormonal changes may in part be secondary ...a cold induced increase in muscular activity. To further elucidate the relationship ...en thermoreception, hormonal secretion and muscular activity we have now studied ...ormonal responses to prolonged swimming at different water temperatures.

Methods

...thy males (age 31 (23–41) years (mean and range) and body weight 73 (65–83) kg) volunteered to be ...ts after being fully informed as to the nature and risk of the experiments. All experiments took place ...swimming flume, in which both water speed and temperature can be accurately regulated (Åstrand and ...sson 1972). Before commencing the actual experiments the subjects underwent a number of practice ...at various water velocities to accommodate themselves to swimming in the flume. Furthermore, on at ...two occasions the subjects had their maximal oxygen uptake ($\dot{V}_{O_2}$ max) determined during breast ...swimming in 27°C water the velocity of which was increased until the swimmer was completely ...sted (Holmér and Bergh 1974). The $\dot{V}_{O_2}$ max achieved by the subjects during graded swimming at ...was 3.74 (2.65–4.27) l min^{-1}. The simultaneously determined pulmonary ventilation and heart rate ...118.6 (109.5–125.4) l min^{-1} and 169 (163–176) beats min^{-1}, respectively.

...actual experiments consisted of breast stroke swimming for 1 h in the flume at a speed calculated ...to require 65 per cent of the previously determined $\dot{V}_{O_2}$ max. At intervals of 1–5 weeks each subject swam ...at water temperatures of 21°C, 27°C and 33°C, respectively. The sequence of water temperatures was varied ...among the subjects.

...Following an overnight fast the subjects reported to the laboratory in the morning. A catheter was ...ted approximately 30 cm into a superficial, cubital vein, and a 2 m long manometer line was attached ...end of the catheter. The manometer line and catheter were filled with physiological saline containing ...m citrate (20 and 130 mM per litre). Following a 15 min rest in the supine position, a blood sample was ...n (test 1). Then a rectal thermistor and an esophageal thermistor (in 3 subjects only) were positioned, ...chest electrodes for the recording of heart rate (HR) were fixed. The temperature in the deltoid and ...a lateralis muscles was measured using needle thermocouples, whereupon the subjects entered the ...and floated for 9 min in calm water supported by a flotation board. A sample of expired air and ...d sample (test 2) were taken near the end of this rest period. During the subsequent 60 min of ...ming blood and gas samples were collected intermittently as depicted in Fig. 1. At the completion of ...m the subjects went quickly out of the water and muscle temperatures were measured again. The ...ects were wiped with a towel and then sat for 15 min at room temperature (18–2?°C). At the end of this ...period the last blood sample (post exercise) was drawn.

...ectal and esophageal temperatures as well as heart rate were continuously monitored by means of ...instrumentation system (Danica Electronic). Oxygen uptake was measured with the Douglas bag method.

...In agreement with previous findings (Holmér 1972) the subjects in the present study had lower values of ...imal oxygen uptake (14) and of maximal heart rate (10) during swimming at 27°C than during ...ycle exercise.

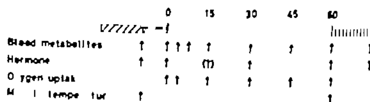


Fig. 1. The sequence of ... a single experiment. The ... indicate the times at which ... and gas were sampled and ... temperatures measured.

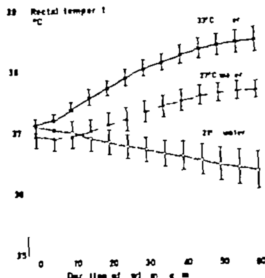
After deproteinization with cold perchloric acid blood was analysed for lactate and glycerol by fluorimetric enzymatic procedures (Lowry and Passonneau 1972). Growth hormone (GH) and ... serum were measured by commercially available solid phase radioimmunoassay kits (... CEA-IRE SORIN respectively). 1 U GH equals 0.5 mg of The first international reference preparation of human growth hormone (66/217) from the National Institute for Biological Standards and Control, Hill, London. The intra assay coefficient of variation of the GH assay was 4 ... at 24 μU·ml⁻¹ ... inter-assay coefficient of variation was 10%. The intra-assay coefficient of variation for the cortisol ... was 13% at ... μg 100 ml⁻¹ and the inter assay coefficient of variation was 15%. Plasma for ... free fatty acids (FFA), glucose, insulin, glucagon and catecholamines was sampled and ... previously described (Galbo *et al.* 1975). ... Insulin and glucagon were measured by radioimmunoassay ... adrenaline and noradrenaline by a double isotope derivative assay. Every subject had all ... hormone carried out in ... single assay run. Statistical evaluation of the data ... was made by ... correlation analysis and by means of the t test for paired comparisons. Differences were considered significant if p values were less than 0.05.

Results

None of the *basal values* (rest₁) differed significantly in the 3 experiments. During ... (rest₂) oxygen uptake and heart rate were higher in the 21°C expts. (0.74 (0.52–1.29) l ... (mean and range) and 73 (54–96) beats min⁻¹ respectively) and lower in the ... expts. (0.33 (0.23–0.39) and 54 (36–67)) than in the 27°C expts. (0.50 (0.32–0.59) and ... (42–78)). At rest₂ noradrenaline concentrations were always higher than at rest₁ ... the concentrations of adrenaline (59%) (Fig. 3), glucagon (14%) (Fig. 5), and lactate ... (Fig. 6) only increased significantly upon flotation in the 21°C expts. The remaining ... did never change during flotation.

Rectal temperature increased significantly during *swimming* in the 33°C water (1.3 ± ... mean and S.E.) and to a smaller extent ($p < 0.05$) in the 27°C water (0.7 ± 0.1°C) but ... creased in the 21°C expts. (0.8 ± 0.3°C) (Fig. 2). In the 3 subjects in whom both ... tures were measured the changes in rectal and esophageal temperature were identical. ... *muscle temperatures* increased significantly during swimming in the 33°C water (2.6 ± ... and 2.9 ± 0.3°C in the deltoid and vastus lateralis muscle, respectively) and to a smaller extent ($p < 0.05$) in the 27°C water (0.5 ± 0.3°C and 2.1 ± 0.4°C). In the 21°C water temperature of the deltoid decreased (1.2 ± 0.3°C) during swimming while that of the ... muscle did not change significantly (+0.6 ± 0.5°C).

The mean *oxygen uptake* was constant throughout prolonged swimming in the different expts. It averaged 2.22 ± 0.18 l min⁻¹ (33°C-expts.), 2.56 ± 0.24 (27°C-expts.) and 2.51 ...



Mean rectal temperatures (±S.E.) during swimming at 3 water tempera-

...expts.), being significantly lowest in the 33°C-expts., and in the 27°C-water amounting ...(59-81)% of the previously determined $\dot{V}_{O_2}$ max in this environment.

...oughout exercise in all expts. the concentrations of *noradrenaline* as well as of ...aline in plasma were significantly higher than at rest (Fig. 3). Compared to the values ...27°C expts. noradrenaline concentrations were significantly higher throughout ...ming in the 21°C expts. (on an average 87%) and at the end of exercise in the 33 C ...(65%). Also the adrenaline concentrations were higher during swimming at 21°C ...at 27°C (71%), the difference being significant between samples obtained at the 30'th ...of swimming. At that time, although significantly lower than in 21°C expts., adrenaline ...ntrations in the 33°C expts., too, were significantly higher (25%) than in the 27°C ... 15 min post exercise the concentrations of catecholamines in the 21°C expts. ...not decreased significantly. However in the other expts., they had declined but were ...higher than at rest. The mean concentration of noradrenaline was significantly higher ... the 33°C expts. than after the 27°C expts.

...ring and after exercise in the 27°C expts. the concentrations of *cortisol* in serum in...ed gradually above values at rest (Fig. 4). In the 33°C expts., compared to the 27°C ...s, the concentrations of cortisol increased more rapidly during the first 30 min of ...ming. Furthermore, the subject who had the highest increase in rectal temperature ...ng exercise (from 37.3 to 39.1°C) also had the highest increase in cortisol concentration ...m 11 µg 100 ml at rest to 32 and 4 at the end of and after exercise, respectively).

...rowth hormone concentrations increased during exercise in 27° and 33°C expts. ...e values at rest, the increase being significant after 60 and 30 min of swimming, ...ectively (Fig. 4). 15 min after exercise in these expts. GH concentrations had ...eased but were still above values at rest. In the 21°C expts., however neither cortisol ... GH changed significantly during or after exercise.

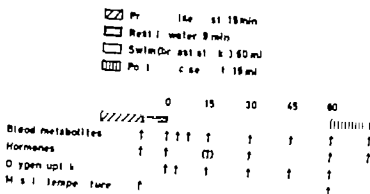


Fig. 1. The sequence of ... a single experiment. The ... indicate the times at which ... and gas ... were sampled and ... temperatures measured.

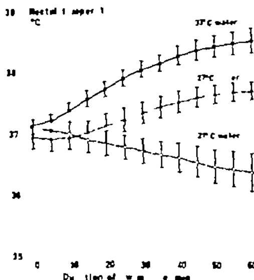
After deproteinization with cold perchloric acid blood was analysed for lactate and ... fluorimetric enzymatic procedures (Lowry and Passo neau 1972). Growth hormone (GH) and ... serum were measured by commercially available sol d phase radioimmunoassay kits ... CEA IRE-SORIN respectively). 1 U GH equals 0.5 mg of The first International reference preparation ... human growth hormone (66/217) from the National Institute for Biological Standards and Control ... H ll, London. The intra-assay coefficient of v riation of the GH assay as 4% at 4 μU ml⁻¹ ... inter-assay coefficient of variation was 10%. The intra-assay coefficient of variation for the ... was 13% at 22 pg 100 ml⁻¹ and the inter-assay coefficient of ariation as 15 ... Plasma for ... free fatty acids (FFA), glucose, insulin, glucagon and catecholamines was sampled and stored as previously described (Galbo et al. 1975), i.e. Insulin and glucagon were measured by radioimmunoassay ... adrenaline and noradrenaline by a double-isotope derivati e assay E ery subject had all ... hormone carried out in a single assay run Statistical evaluation of the data as made by ... correlation analysis and by means of the t-test for paired comparisons. Differences were considered significant if p v lues were less than 0 05.

Results

None of the *basal values* (rest) differed significantly in the 3 experiments. During ... (rest$_2$) oxygen uptake and heart rate were higher in the 21 C expts. (0 74 (0.52 1.26) l ... (mean and range) and 73 (54–96) beats min ¹ respectively) and lower in the ... expts. (0.33 (0.23–0 39) and 54 (36–67)) than in the 27°C expts. (0.50 (0.3?–0.59) and (4?–78)) At rest noradrenaline concentrations were always higher than at rest, ... the concentrations of adrenaline (59 %) (Fig. 3) glucagon (14%) (Fig. 5), and lactate (Fig. 6) only increased significantly upon flotation in the 21 C expts. The remaining ... did never change during flotation

Rectal temperature increased significantly during *swimming* in the 33 C water (1.3 ... mean and S E.) and to a smaller extent ($p < 0.05$) in the 27°C water (0.7 ± 0 1°C) ... creased in the 21°C expts (0 8 + 0 3 C) (Fig. 2). In the 3 subjects in whom both temperatures were measured the changes in rectal and esophageal temperature were identical ... *muscle temperatures* increased significantly during swimming in the 33 C water (2.6 ... and 2.9 ± 0 3°C in the deltoid and vastus lateralis muscle, respectively) and to a smaller extent ($p < 0.05$) in the 27°C water (0 5 ± 0.3 C and 2.1 ± 0.4 C). In the 21°C water temperature of the deltoid decreased (1 2 ± 0 3 C) during swimming while that of the ... muscle did not change significantly (+ 0 6 ± 0.5°C)

The mean *oxygen uptake* was constant throughout prolonged swimming in the different expts. It averaged 2.22 ± 0 18 l min (33°C-expts) 2.56 ± 0.24 (27°C-expts.) and 2.51 ...



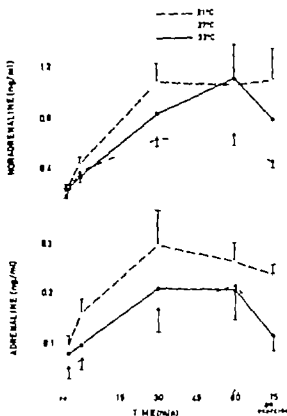
Mean rectal temperatures (±S.E., ...ring swimming at 3 water tempera-

...expts.), being significantly lowest in the 33°C-expts., and in the 27°C-water amounting (59-81)% of the previously determined $\dot{V}_{O_2}$ max in this environment.

...oughout exercise in all expts. the concentrations of *noradrenaline* as well as of ...line in plasma were significantly higher than at rest, (Fig. 3). Compared to the values ... 27 C expts. noradrenaline concentrations were significantly higher throughout ...ming in the 21°C expts. (on an average 87%) and at the end of exercise in the 33 C ... (65%) Also the adrenaline concentrations were higher during swimming at 21°C ...at 27 C (71%), the difference being significant between samples obtained at the 30'th ...f swimming. At that time, although significantly lower than in 21°C expts., adrenaline ...ntrations in the 33°C expts., too, were significantly higher (25%) than in the 27°C ... 15 min post exercise the concentrations of catecholamines in the 21°C expts. ...not decreased significantly. However in the other expts., they had declined but were ...higher than at rest. The mean concentration of noradrenaline was significantly higher ... the 33°C expts. than after the 27°C expts.

...ring and after exercise in the 27°C expts. the concentrations of *cortisol* in serum in...ed gradually above values at rest (Fig. 4). In the 33°C expts., compared to the 27°C ...s., the concentrations of cortisol increased more rapidly during the first 30 min of ...ming. Furthermore, the subject who had the highest increase in rectal temperature ...ng exercise (from 37.3 to 39.1°C) also had the highest increase in cortisol concentration ...n 11 μg 100 ml⁻¹ at rest to 32 and 42 at the end of and after exercise, respectively).

...*Growth hormone* concentrations increased during exercise in 27° and 33°C expts. ...e values at rest, the increase being significant after 60 and 30 min of swimming, ...ectively (Fig. 4). 15 min after exercise in these expts. GH concentrations had ...eased but ...ere still above values at rest. In the 21°C expts., however neither cortisol ... GH changed significantly during or after exercise.



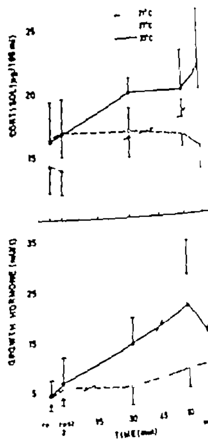
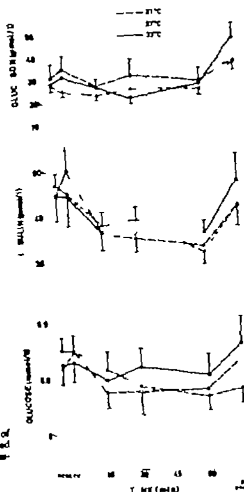


Fig. 3 Mean concentrations (± S.E., n = 6) of catecholamines in plasma obtained at rest and during swimming at 3 water temperatures.

Fig. 4 Mean concentrations (± S.E., n = 6) of cortisol and growth hormone in serum obtained at rest and during swimming at 3 water temperatures.

At the 15'th and 30'th min of swimming in the 27° and 33°C expts, respectively plasma glucagon concentrations had significantly decreased below values at rest (Fig. 5). Subsequently plasma glucagon concentrations increased and were 15 min post exercise significantly higher than at any other time in these expts. In the 21°C expts, glucagon concentrations did not change significantly during or after exercise. Throughout exercise *insulin* concentrations in plasma were always significantly below values at rest (Fig. 5). After exercise insulin concentrations increased significantly but remained in the 21°C expts, significantly below rest values. *glucose* concentrations in plasma decreased slightly in all expts. during swimming, the decrease, however being significant only in the 27°C expts. at the end of swimming (Fig. ... Insulin glucose molar ratios were uniformly depressed during swimming at the different water temperatures. In the 21°C expts, decreases in glucose concentrations were inversely related to the simultaneous decreases in rectal temperature ($r = -0.90$, $p < 0.01$), and in the 33°C expts. decreases in glucose concentrations were inversely related to the simultaneous increases in rectal temperature ($r = -0.73$, $p < 0.1$). After exercise in the 33°C expts



Mean concentrations (±S.E. n=6) of ..., insulin and glucose in plasma obtained ... and during swimming at 3 water temperatures.

... glucose concentration increased significantly above the mean concentration at the ... of swimming but remained in 27°C expts. significantly below preexercise values.

... carbohydrate combustion rate (calculated from O_2 uptake and R value (respiratory ... ratio) measurements) did not change significantly with the duration of swimming. ... ded to be higher in the 21°C expts. than in the 33°C expts. (representing on an average ... 0-68) and 41 (29-59)% (p 0.1), respectively of total energy output per min) and was ... mediary in the 27°C expts. (53 (34-61)%). The concentrations of *FFA* in serum ... ys decreased at the beginning of swimming but subsequently they increased and were ... *glycerol* concentrations—at the end of and after swimming significantly above pre- ... the values (Fig. 6). At identical times in the different expts. the concentrations of FFA ... of glycerol ere identical. I 1°C and 27°C expts. the blood *lactate* concentrations ... hed a maximum early in exercise and were in the 15'th min in these expts. significantly

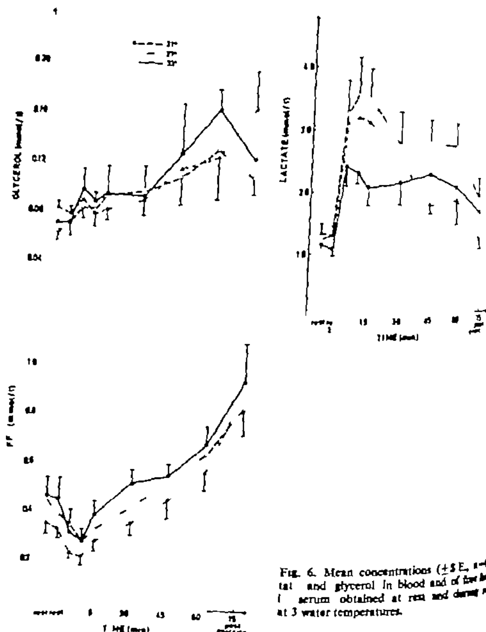


Fig. 6. Mean concentrations (±S E, n=6) of lactate and glycerol in blood and of free fatty acids in serum obtained at rest and during swimming at 3 water temperatures.

higher than in 33°C expts (Fig. 6). Throughout swimming in 21 C and 33°C expts and in 33 C expts. also after exercise, lactate concentrations were higher than preexercise values.

In the 5'th min of swimming the mean *heart rates* were identical in the 3 expts (131 (33 C) 131 ±5 (27 C) and 127 ±7 (21 C)) Subsequently heart rate increased significantly more at 33 C than at 27°C (to 143 ±8 and 137 ±5 respectively in the 60'th min) while no change occurred at 21 C. Accordingly throughout exercise the oxygen pulse (O₂ uptake heart rate) was significantly lower in the 33 C expts. than in the other expts. and in the final part of exercise also lower in 27°C expts. than in 21°C expts. The *subjective experience* of swimming at the different water temperatures varied *from individual to individual*, but in general the subjects felt cold in the 21 C water *and enclosed in heat* in the 33°C water and found it more difficult to complete the swim at *these water temperatures* than at

Discussion

nent study lends support to the concept that the rise in body temperatures normally
d during exercise enhances the exercise-induced increases in the plasma concentra-
f noradrenaline, cortisol, growth hormone and glucagon. A wide range of deep as
superficial body temperatures were attained. Skin temperature has previously been
to be closely related to water temperature during swimming (Nadel *et al.* 1974) and
erved changes in rectal (Fig. 2), esophageal and muscle temperatures, too, varied in
ith the temperature of the water. The changes in rectal, esophageal and muscle
atures almost covered the range of changes in these temperatures found during sub-
al exercise in a neutral thermal environment (Saltin and Hermansen 1966). When in
ater the external conditions for heat dissipation were worst the increases in plasma
enaline concentrations and heart rate during swimming were larger than those
ng at 27°C. Correspondingly the exercise induced noradrenaline excretion in the
has been shown to increase in a hot environment (Maher *et al.* 1972), and the
trations of catecholamines in plasma are higher during running, when secretion of
is impeded by atropine (Galbo *et al.* 1977). These findings are in accordance with the
ption (Rowell 1974) that heat stress in addition to exercise elicits an increased sym-
ic nervous outflow which in turn increases heart rate and decreases visceral blood
e and flow thus making possible maintenance of cardiac output and muscular blood
n the face of increased skin blood volume and flow. It should be emphasized,
er that the plasma noradrenaline level is only an indirect measure of sympathetic
s activity. Certainly noradrenaline levels in the venous effluent have been shown to
rectly related to the sympathetic nervous activity in an organ (Yamaguchi, de
plain and Nadeau 1975). But it is at the present time not possible to exclude the
ilities that the relationship between impulse frequency and transmitter overflow to
ood stream is different in different organs and dependent on the physiological milieu
ding temperature) at the adrenergic nerve endings (Langer 1974, Starke 1977).
ermore the elimination of noradrenaline from plasma may vary during different
ological conditions. Thus identical overall activities in the sympathetic nervous system
in theory during different patterns of regional nervous activity and during different
ological conditions lead to different noradrenaline concentrations in arterial plasma.
ring swimming the concentrations of cortisol and growth hormone in serum only in-
ed in expts. in which body temperature increased (Fig. 4). Furthermore only in such
did the post-exercise concentration of glucagon in plasma exceed the preexercise values
5). Available information about the temperature dependence of glucagon secretion
rce. It solely stems from studies of acute infections in which an elevated body tem-
ure is accompanied by plasma glucagon concentrations which relative to glucose
ntrations are increased in the fasting state as well as during intravenous glucose
rance tests (Rayfield *et al.* 1973, Rocha *et al.* 1973, George *et al.* 1974). In such studies
an increased plasma cortisol concentration (George *et al.* 1974), increased urinary free
ol excretion (Rayfield *et al.* 1973) and an increased serum growth hormone concen-
on (Rayfield *et al.* 1973) may be found. However the increase in body temperature

may not be essential for the hormonal changes during infection. This is illustrated by the fact that the rise in plasma concentrations of growth hormone (Glick 1968) and [illegible] colds (Collins and Weiner 1968) seen after injection of bacterial pyrogen may occur in the absence of a rise in body temperature. Yet the opinion that increased body temperature enhances cortisol and growth hormone secretion is also based on other kinds of [illegible] Thus cortisol secretion is enhanced when hyperthermia is induced at rest by [illegible] or exposure to a hot environment (Collins and Weiner 1968), and the rise in growth hormone concentration during bicycle exercise has actually been shown to correlate with the [illegible] rectal temperature (Buckler 1973) It is tempting to propose that increased body temperature also directly stimulates glucagon secretion. Glucose abundance within the pancreatic cells is possibly the single most potent physiologic glucagon suppressor (Unger and Orci 1976) By increasing the metabolism of glucose an elevated A cell temperature [illegible] decrease the suppressive effect on glucagon secretion of prevailing plasma glucose concentrations In accordance with this hypothesis glucagon concentrations in 33°C expts in the present study were significantly increased shortly after exercise even though [illegible] glucose concentrations in these expts. never decreased significantly below [illegible] Moreover in two previously studied subjects glucagon concentrations did not [illegible] during prolonged running in a hot environment in spite of an increase in blood [illegible] concentrations (Rowell *et al* 1968) Since catecholamines may enhance A cell [illegible] (Unger and Orci 1976) it could be argued that the temperature dependence of glucagon secretion during exercise is indirect being due to temperature dependent variations in sympathetic activity However glucagon concentrations did not increase in 21°C [illegible] (Fig 5) in which sympathetic activity was at least as intense as in 33 C expts. (Fig 3) [illegible] fact that an increase in the plasma glucagon concentration when at all present [illegible] detectable late in the present expts. is consistent with observations during [illegible] running of similar intensity and has tentatively been ascribed to increased hepatic glucagon extraction as a consequence of decreased splanchnic blood flow during exercise (Galbo *et al* 1976)

Immersion of man in 10°C water induces an increase in the concentration of noradrenaline in plasma which is correlated with both the simultaneous increase in metabolic rate caused by shivering and with the degree of central hypothermia (Johnson *et al* 1977). If the [illegible] subsequently is heated (to approximately 40°C) noradrenaline concentration and [illegible] metabolic rate decreases to basal levels even though core temperature continues [illegible] (Johnson *et al* 1977). From these findings it is not possible to decide whether increased release of noradrenaline in response to cold is a direct consequence of [illegible] reception or secondary to activation of skeletal muscle. However the present study [illegible] indicates that increased sympathetic activity can be directly elicited from cold receptors During swimming in 21°C and 27°C water oxygen uptakes were identical but the concentrations of catecholamines in plasma were markedly higher in 21°C expts in which [illegible] temperatures were lower than in 27°C expts (Fig. 3). A close correlation has previously been found between plasma noradrenaline and heart rate during exercise of more than [illegible] intensity (Christensen and Brandsborg 1973) This correlation probably reflects the [illegible] principle that after vagal withdrawal during exercise increased sympathetic nervous [illegible]

heart to increase its rate is accompanied by a proportional increase in sympathetic otor outflow to visceral organs (Rowell 1974). As expected (Rowell 1974) drift of rate during swimming was eliminated in 21°C expts, in which body temperatures did crease. Nevertheless the concentrations of catecholamines in these expts. were high swimming leading to a higher ratio between plasma noradrenaline and heart rate 27°C expts. This finding questions the predictability of visceral vasomotor tone from rate measurements but may rather indicate that during exercise at low body temres sympathetic nervous outflow to skin vessels and to the adrenal medulla increases, y securing low skin blood flow and volume in co-operation with the direct effects skin temperature (Iriki *et al.* 1971 Riedel *et al.* 1972, Gale 1973 Rowell 1974). During sion in 21 C water the concentrations of growth hormone and cortisol in serum did crease above basal values, neither during mild shivering in the preexercise period nor metabolic rate during swimming was nearly twice as high as previously found during nal shivering (Molnar 1946). These findings indicate that previously observed increases concentrations of these hormones during cold stress are not exclusively due to ing. Furthermore, if at all such hormonal responses can be elicited in humans, in to do so one has to provoke lower body temperatures than those attained in the nt study

pothermia has in the fasting state been found to be associated with unchanged (George 1974) or decreased (Rayfield *et al.* 1973, Frascella *et al.* 1977) insulin glucose molar whereas relative hyperinsulinemia has been found after glucose loading (Rayfield 1973 George *et al.* 1974). I markedly shivering adrenodemedullated dogs exposed 21°C ambient temperature rectal temperature decreased 0.8°C (Forichon *et al.* 1977). the dogs a relative hypoinsulinemia was found during hyperglycemia and tentatively red to an inhibition of insulin secretion by a direct cold-induced stimulation of the atic sympathetic nerves (Forichon *et al.* 1977). I the present study plasma insulin ntrations always decreased markedly during swimming (Fig. 5), the insulin, glucose r ratio being uniformly depressed at the different water temperatures. Thus this study not unveiled any significant influence of body temperatures on insulin secretion during se and accordingly does not refute the supposition that the increase in -adrenergic ity accompanying activation of skeletal muscle is sufficient to explain the depression ulin secretion found previously during cold stress (Galbo *et al.* 1977).

agreement ith previous findings (Nadel *et al.* 1974) oxygen uptake in the present study slightly lower during swimming in 33°C water than during swimming at the colder er temperatures. Furthermore, maximal oxygen uptake has previously been shown in subjects to be directly related t core temperature within the range of core temperatures rved in the present study (Holmér and Bergh 1974). Accordingly the relative work load ($\dot{V}_{O_2}$, $\dot{V}_{O_2}$ max) possibly aried inversely with water temperature in the latter half e swimming period. If so differences in relative work load may ha influenced the erences in hormonal secretion between the different expts. and may furthermore, lain the tendency to higher relative carbohydrate combustion rate during swimming in er ater Whereas the higher blood lactate concentrations in 21°C expts. are compatible an enhancement of muscular glycogenolysis by the higher plasma adrenaline con-

centration in these expts., identical concentrations of FFA and glycerol and calculated rates of fat combustion in the different expts. do not indicate any enhancement of lipolysis by the higher sympathoadrenomedullary activity in the colder water. Even during swimming in 21°C water lipolysis may have been limited by a low skin blood [illegible]

In conclusion the present study supports the concept that the rise in body temperature normally observed during exercise enhances the exercise-induced increases in the plasma concentrations of noradrenaline, cortisol growth hormone and glucagon. Furthermore the study indicates that decreased body temperatures may elicit increased concentrations of noradrenaline and adrenaline in plasma as a direct consequence of thermoregulation. Activation of shivering may account for previously observed decreases in insulin concentration during cold stress but do not explain increases in cortisol and growth hormone concentrations.

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Release of ³H noradrenaline from the rat vas deferens under various in vitro conditions

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Abstract

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The release of ³H-(−)-noradrenaline (NA) from rat vas deferens *in vitro* was examined under various experimental conditions. It was found that in normal and reserpinized vas deferens the release of ³H by (+)-amphetamine (5 × 10⁻⁶ M) or low external Na (26 mM) was antagonized by imipramine and desipramine inhibitors of the NA uptake but was not dependent on the presence of Ca^{2+} in the medium and was not antagonized by the potent local anaesthetic agent bethoxycaine. The release by veratridine in reserpinized tissue was antagonized by the uptake inhibitors but was in normal only partially inhibited in presence of Ca^{++} but almost completely in absence of Ca^{++}. The release by K (117 mM) + low Na (26 mM) in normal tissue was dependent on the presence of Ca^{++} and antagonized by the muscarinic agonists carbacholine and metacholine and by high concentrations of desipramine. In the reserpinized vasa the corresponding release was not dependent on Ca^{++} and was not antagonized by the muscarinic agents but was inhibited by high concentrations of desipramine.

Key words: Noradrenaline release, vas deferens, rat, desipramine, imipramine, methiodide

The impulse-propagated release of noradrenaline (NA) from sympathetic nerve terminals is Ca^{++} dependent and appears to occur with an exocytotic mechanism (Douglas 1968, Smith and Winkler 1972). Under certain *in vitro* conditions a Ca^{++} independent NA release mechanism has been demonstrated (Paton 1973). This release is antagonized by cocaine and desipramine and seems to be a carrier-mediated transport of NA out from the nerve terminals.

In previous studies we found that ³H-bretylium was released from the rat vas deferens by a Ca^{++} independent mechanism very similar to the proposed carrier-mediated release of NA (Ross and Kelder 1976 a, b, 1977). Since bretylium appears to be accumulated in the NA nerve terminals by the membranal NA carrier (Ross and Gosztonyi 1975, Ross and Kelder 1976 a) it is possible that it is also released by a NA transport mechanism. In order to further establish the similarity between the carrier-mediated release of NA and bretylium in the same organ we have now examined the release of ³H NA in the rat vas deferens under various *in vitro* conditions.

Materials and methods

[illegible] vas deferens weighing about 20 mg from 170–250 g [illegible] hite rats (Sprague Dawley) [illegible] ere used. The [illegible] ion medium consisted of 117 mM NaCl, 5.4 mM KCl, 0.6 mM $MgSO_4$, 2.6 mM $CaCl_2$, 1.1 mM [illegible] O_4, 25 mM $NaHCO_3$, 5.4 mM glucose, 0.25 mM pargyline, 1.1 mM ascorbic acid and 0.13 mM [illegible] Na_2. In some experiments NaCl was substituted with 250 mM sucrose (low Na^+) or with 117 mM [illegible] h K [illegible] low Na^+).

[illegible] sa were pre-incubated for 15 min in the ordinary incubation medium in order t[illegible] inhibit the mono-[illegible] oxidase (MAO) activity and were loaded [illegible] ith ³H-NA ($1 \cdot 10^{-7}$ M) by incubation for 1 hour at 37° [illegible] sa were blotted on filter paper and washed three times in fresh incubation medium. The vasa were [illegible] ed in fresh medium for 10 min at 37° in order to [illegible] ash out loosely bound ³H NA. Each piece of [illegible] ssue was then transferred to [illegible] glass stoppered centrifuge tube containing 0.5 ml of the appropriate [illegible] ion medium and the additives to be studied. The tubes were gassed [illegible] ith 93.5% O_2 + 6.5[illegible] CO_2. [illegible] bation [illegible] as performed for 10 min, the vasa [illegible] ere blotted on filter paper [illegible] eighed and transferred [illegible] ting vials containing 1.0 ml of Soluene-350 (Packard). After one hour at 65° the tissue was dissolved [illegible] ml of the scintillation liquid (Permablend III, Packard, in toluene) were added. Six ml of Instagel [illegible] rd) were added to the incubation medium. The radioactivity was counted in [illegible] liquid spectrometer [illegible] rds added to the medium and tissue dissolved in Soluene-350 were determined in each experiment. [illegible] mount of radioactivity released into the medium was calculated and expressed as per cent of the [illegible] tivity at the start of the release experiment ([illegible] the sum of the radioactivity in tissue and medium). [illegible] ome experiments the amounts of intact [illegible] H NA, of ³H-labelled acid and neutral metabolites, and of [illegible] methanephrine in the incubation medium [illegible] ere determined by chromatography on [illegible] weak acid anion [illegible] ge resin (Amberlite CG50 Type 1 (100–200 mesh), Na [illegible] 30 [illegible] 5 mm columns), prepared according [illegible] Malherbe (1968). Immediately after incubation 2 ml of 0.1N HCl containing 0.05 [illegible] EDTA Na_2 [illegible] added to the media [illegible] hich were stored at −18°C until the assay [illegible] than one week. 10 ml of 0.1[illegible] [illegible] Na_2, 1 ml of 0.1 N sodium phosphate buffer pH 6.5 were then added and pH was brought t[illegible] 6.0 [illegible] by addition of 0.01 N NaOH. 10 ml of this solution were run through the resin columns and 1 ml [illegible] ken for determination of total radioactivity. The effluent contained the acid and neutral metabolites. [illegible] washing [illegible] ith 2 [illegible] 10 ml of distilled [illegible] ater ³H-NA [illegible] as eluted with 10 ml of 4[illegible] boric acid. The columns [illegible] washed [illegible] ith 10 ml of distilled water and ³H-normetanephrine was eluted with 10 ml of 4 N NH_4OH. [illegible] dioactivity in 5 ml of these fractions was measured after addition of 5 ml of Instagel.

[illegible] pounds: ([illegible])-Noradrenaline-7 [illegible] H (specific activity 3.2 Ci/mmol) [illegible] as purchased from NEN GmbH, [illegible] Dreieich, West Germany. Veratridine [illegible] as bought from EGA-Chemie K.G., Steinheim, Albuch, [illegible] Germany and pargyline from Saber Laboratories Inc., Morton Grove, Ill., U.S.A. Imipramine [illegible] iodide [illegible] as synthesized from imipramine, [illegible] hich like desipramine [illegible] ere gifts from Ciba-Geigy AG. [illegible] ocaine hydrochloride (Malicaine [illegible]) [illegible] as [illegible] gift from Les Laboratoires Millot, Arcueil, France.

Results

[illegible] course in reserpinized tissue

[illegible] spontaneous release measured as the radioactivity in the medium was markedly tem-[illegible] ture dependent (Fig. 1). The release at 37° followed a biphasic course with a rapid [illegible] al phase during the first 5–10 min followed by a second slow phase ($T_{\frac{1}{2}}$ ~ 2.5 ± 0.1 hours, [illegible] 5). Extrapolation of the second phase to zero time estimates the amount released in the [illegible] al phase (10 [illegible] 6 [illegible] n [illegible] 6, of the total radioactivity).

[illegible] Imipramine (3 [illegible] 10[illegible] M) and imipramine methiodide (2.4 [illegible] 10^{-4} M) accelerated the [illegible] ntaneous release but antagonized the release evoked by ([illegible])-amphetamine (5 [illegible] 10^{-4} M) [illegible] veratridine (5 [illegible] 10^{-4} M) (Fig. 1). High concentration of K[illegible] (75 mM), low concentra-[illegible] of Na[illegible] (26 mM) and high K[illegible] (117 mM) + low Na[illegible] (26 mM) induced marked release [illegible] ch like that evoked by amphetamine and veratridine was almost completely abolished [illegible])

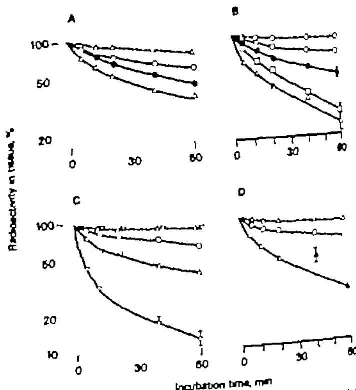


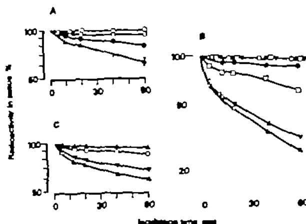
Fig. 1 Time courses of the release of H NA evoked by various means from reserpinized rat vas deferens measured as the radioactivity remaining in the tissue. Each value is the mean ± S.E. (vertical bars hidden in the symbols) of 4 determinations. The incubations were performed at 37° (dotted lines). Control (O). A Desipramine 3 × 10⁻ M (●), (+)-Amphetamine, 5 × 10⁻ M (Δ), imipramine methiodide, 2.3 10⁻ M (●), veratridine 5 × 10⁻ M (Δ), imipramine methiodide, 2.3 veratridine 5 10⁻ M (□). C. Hypertonic K⁺ 75 mM (Δ), high K (117 mM) + low Na (26 mM). D Low Na (26 mM substituted with 0.25 M sucrose) (Δ).

Time course in normal tissue

The spontaneous release of H-noradrenaline from the normal (non-reserpinized) vas deferens was much less than that from the reserpinized tissue (Fig. 2). Thus, approximately 10% of the radioactivity accumulated was released during one hour's incubation at 37°. Desipramine (3 10⁻ M) and imipramine methiodide (2.4 10⁻ M) only slightly accelerated this release (+)-Amphetamine (5 10⁻ M), high external concentration of K (75 mM), low Na (26 mM) and particularly high K (117 mM) + low Na (26 mM) and veratridine (5 10⁻ M) evoked marked release at 37° but not at 0°

Dose response at the release evoked by (+)-amphetamine

The concentration curve of the release of H NA evoked by (+)-amphetamine in the reserpinized tissue showed an optimal effect at the concentration between 1 10⁻ 1 M at which about 40 per cent of the accumulated ³H NA was released (Fig. 3). This curve was almost identical with that observed for the release of ³H-bretylium (Ross and Kelder 1976 a) and for that of H NA in normal vas deferens (Fig. 3) However the amount released in the normal preparation was only one fourth of that in the reserpinized vas. Fifty per cent of the optimal release was obtained at about 5 10⁻ M of (+)-amphetamine in all three cases.



2. Time courses of the release of ³H NA evoked by various means from rat vas deferens measured as radioactivity remaining in the tissue. Each value is the mean ± S.E. (vertical bars often hidden in the symbols) of 4 determinations. Control (○). A. Desipramine, 3 · 10⁻⁷ M (●), (+)-amphetamine, 5 · 10⁻⁴ M. B. Imipramine methiodide, 2.3 · 10⁻⁴ M (●), hypertonic (75 mM) K (□), high K (117 mM)+low Ca mM) (▽), veratridine, 5 · 10⁻⁴ M (△). C. Low Na⁺ (26 mM, substituted with 0.25 M sucrose) (△), ... methiodide 2.3 · 10⁻⁴ M + low Na⁺ (▽).

...paration of H-NA from labelled metabolites

...order to verify that the radioactivity released into the medium consisted mainly of H-NA the labelled neutral and acid metabolites and ³H-normetanephrine (NM) were separated from ³H-NA on Amberlite CG50 columns (Table I). No detectable amounts of neutral and acid metabolites were released from the normal tissue and low concentrations were found in the medium of the reserpinized vasa. The MAO inhibition was accordingly effective. The NM values were low in the medium of the normal tissue but constituted a considerable part of the spontaneous released radioactivity from the reserpinized tissue. However it was only slightly increased by the means which strongly released H NA into the medium. Hence the evoked release of radioactivity was mainly H NA.

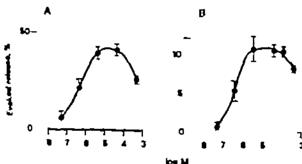


Fig. 3. Release of ³H-NA from reserpinized (A) and normal (B) rat vas deferens evoked by various concentrations of (+)-amphetamine and measured as the radioactivity released into the medium in per cent of the total radioactivity in the tissue at the start of the incubation. Each value is the mean ± S.E. (vertical bars) of 4 determinations. The incubation time 10 min.

TABLE I Amounts of ^{3}H noradrenaline (NA), ^{3}H-normetanephrine (NM) and ^{3}H-acid + neutral metabolites (Acid) released from the rat vas deferens under various *in vitro* conditions. The vasa were labelled with ^{3}H NA and the release of ^{3}H NA and ^{3}H-metabolites in the medium during 10 min incubation was determined The metabolites were separated from NA on Amberlite CG50 columns. Each value is the mean ± S.E. of 4 determinations. The neutral and acid metabolites in the normal tissue were below detectable amounts.

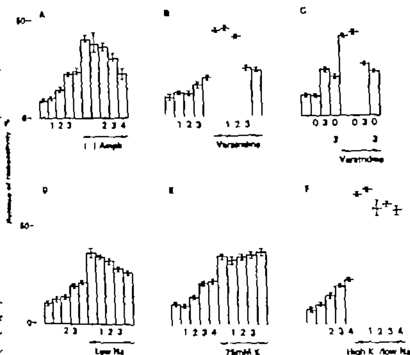
Treatment	Normal tissue		Reserpinized tissue		
	^{3}H NA	^{3}H NM	^{3}H NA	^{3}H NM	^{3}H Acid
	pmol/g wet tissue ± S.E.				
Control	64 ± 14	9 ± 2	64 ± 5	40 ± 5	5 ± 4
(+)-Amphetamine 5 × 10^{-6} M	190 ± 28	9 ± 1	430 ± 6	53 ± 6	9 ± 1
Low Na (sucrose)	137 ± 21	10 ± 2	273 ± 10	72 ± 9	11 ± 1
High K$^+$ + low Na	700 ± 50	6 ± 4	657 ± 14	63 ± 7	10 ± 1
Veratridine 5 × 10^{-6} M	629 ± 31	2 ± 2	—	—	—

Antagonism of the evoked release by uptake inhibitors

In a series of experiments the antagonism of the evoked release by various compounds was examined To avoid the initial spontaneous release the vasa were pre-washed for 10 min at 37 The release of radioactivity during a further 10 min period was measured Imipramine methiodide was used besides desipramine as an inhibitor of the membrane NA transport, since it as a quaternary ammonium derivative can be expected to preferably act on the outside of the membranes which minimizes intraneuronal interactions. Bethoxycaine was used as a potent membrane stabilizing agent which has weak inhibitory action on the noradrenaline transport (Ross and Kelder 1976 b)

Imipramine methiodide antagonized the release of radioactivity evoked by (+)-amphetamine (5 × 10 M) and low external Na (26 mM) in reserpinized (Fig. 4) and normal vasa (Fig. 5) The release by veratridine (5 × 10 M) was only partially (15%) antagonized in the normal tissue but almost completely antagonized by imipramine methiodide in the reserpinized vasa These findings agree with those previously obtained with desipramine (Ross and Kelder 1976 b) The release evoked by high external K (75 mM), low external Na (26 mM) and high K (117 mM) low Na (26 mM) was not apparently antagonized by imipramine methiodide (Fig. 4 and 5) Since high concentrations of imipramine methiodide itself caused significant release particularly in the reserpinized tissue, a partial antagonism is possible, if additive effects are assumed.

Desipramine had similar effects but was considerably more potent than imipramine methiodide (Table II), which is in accord with their potencies as uptake inhibitors (IC in the rat vas deferens was 1 1 × 10 M for desipramine and 2.3 × 10^{-6} M for imipramine methiodide) Large concentrations of desipramine antagonized the release of radioactivity evoked by high K + low Na in the normal and reserpinized tissues (Fig. 6). These concentrations were 100 times larger than those antagonizing the veratridine evoked release in reserpinized vasa but equal to those effective against veratridine in normal tissue. As shown in Table II the two uptake inhibitors antagonized the release evoked by (+)-amphetamine, low Na and veratridine in reserpinized vasa at the same concentrations as they inhibited the accumulation of ^{3}H NA



4 Antagonism by imipramine methiodide of the release of ³H-NA from reserpinized rat vas deferens led by various means. The concentrations of imipramine methiodide were: 1 = 2.4 10^{-7} M, 2 = 2.4 M, 3 = 2.4 10^{-6} M, 4 2.4 10^{-5} M. A. ( )-Amphetamine, 5 10^{-6} M B. Veratridine, 5 10^{-5} M Veratridine 5 10^{-5} M, 0 absence of Ca^{++} and presence of 1 mM EGTA in the incubation medium. Low Na^{+} (26 mM) in the medium (substituted with 0.25 M sucrose). E. Hypertonic K (75 mM) in the dium. F High K (117 mM) low Na^{+} (26 mM) in the medium. ● denotes significant (p 0.05) difference from the control value (= the releasing agent alone).

Bethoxycaine The local anaesthetic agent bethoxycaine did not antagonize the release evoked by ( )-amphetamine (5 10^{-6} M), low external Na (26 mM) and high external (75 mM) in normal or reserpinized tissue (Table III) However the release evoked by veratridine was previously shown to be completely antagonized by bethoxycaine (Ross & Kelder 1976 b). The release evoked by high K + low Na in normal tissue was slightly %) but significantly antagonized by bethoxycaine (Table III).

Ca^{++} dependence of the release In order to differentiate between exocytotic and carrier mediated release Ca was omitted in the incubation medium to which was added 1 mM EGTA to chelate any remaining Ca The release by ( )-amphetamine and that by low Na were not influenced by omission of Ca^{++} (Table IV) In the normal tissue the release by high K and by high K low Na were almost completely antagonized by omission of Ca^{++} The release by veratridine in normal tissue was partially (40%) antagonized by omission of Ca^{++} (Fig. 5 C). Addition of imipramine methiodide to the Ca^{++} free medium completely counteracted the veratridine evoked release.

In the reserpinized tissue the release by high K low Na was decreased (1%) by omission of Ca^{++} (Table IV). No effect was obtained on the ³H-NA release evoked by the other means studied.

TABLE I Amounts of ^{3}H noradrenaline (NA), ^{3}H-normetanephrine (NM) and ^{3}H-acid + neutral metabolites (Acid) released from the rat vas deferens under various *in vitro* conditions. The vasa were incubated with ^{3}H NA and the release of ^{3}H NA and ^{3}H-metabolites in the medium during 10 min incubation was determined. The metabolites were separated from NA on Amberlite CG50 columns. Each value is the mean ± S.E. of 4 determinations. The neutral and acid metabolites in the normal tissue were below detectable amounts.

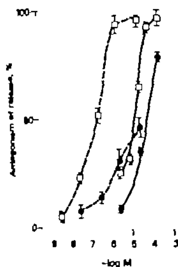
Treatment	Normal tissue		Reserpinized tissue		
	^{3}H NA	^{3}H NM	^{3}H NA	^{3}H-NM	^{3}H-Acid
	pmol/g wet tissue ± S.E.				
Control	64±14	9±2	64± 5	40±5	5±4
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Low Na (sucrose)	137± 1	10±2	273±10	7?±9	11 1
High K$^+$+low Na	700±50	6±4	657±14	63±7	10 1
Veratridine 5 × 10^{-6} M	629±31	2±2	—	—	—

Antagonism of the evoked release by uptake inhibitors

In a series of experiments the antagonism of the evoked release by various compounds was examined. To avoid the initial spontaneous release the vasa were pre-washed for 30 min at 37°. The release of radioactivity during a further 10 min period was measured. Imipramine methiodide was used besides desipramine as an inhibitor of the membrane NA transport since it as a quaternary ammonium derivative can be expected to preferably act on the outside of the membranes which minimizes intraneuronal interactions. Bethoxycaine was used as a potent membrane stabilizing agent which has weak inhibitory action on the noradrenaline transport (Ross and Kelder 1976 b).

Imipramine methiodide antagonized the release of radioactivity evoked by (+)-amphetamine (5 × 10^{-6} M) and low external Na (26 mM) in reserpinized (Fig. 4) and normal (Fig. 5). The release by veratridine (5 × 10 M) was only partially (15%) antagonized in the normal tissue but almost completely antagonized by imipramine methiodide in the reserpinized vasa. These findings agree with those previously obtained with desipramine (Ross and Kelder 1976 b). The release evoked by high external K (75 mM), low external Na (26 mM) and high K (117 mM) low Na (26 mM) was not apparently antagonized by imipramine methiodide (Fig. 4 and 5). Since high concentrations of imipramine methiodide itself caused significant release particularly in the reserpinized tissue, a partial antagonism is possible, if additive effects are assumed.

Desipramine had similar effects but was considerably more potent than imipramine methiodide (Table II) which is in accord with their potencies as uptake inhibitors (K in the rat vas deferens was 1.1 × 10 M for desipramine and 2.3 × 10^{-6} M for imipramine methiodide). Large concentrations of desipramine antagonized the release of radioactivity evoked by high K + low Na in the normal and reserpinized tissues (Fig. 6). These concentrations were 100 times larger than those antagonizing the veratridine evoked release in reserpinized vasa but equal to those effective against veratridine in normal tissue. As shown in Table II the two uptake inhibitors antagonized the release evoked by (+)-amphetamine, low Na and veratridine in reserpinized vasa at the same concentrations as they inhibited the accumulation of ^{3}H NA.



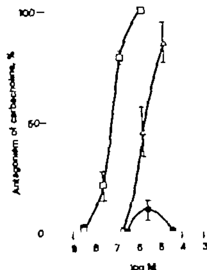


Fig. 6 Fig. 7

6. Antagonism by various concentrations of desipramine on the release of ³H-NA evoked by veratridine 10^{-4} M (□) and high K (117 mM) + low Na (26 mM) (●) in nontreated (whole lines) and reserpinized (lines) rat vas deferens. The incubation time was 20 min in the veratridine experiments and 10 min with high K + low Na⁺. Each value is the mean ± S.E. (vertical bars) of 4 determinations.

7. Dose response curves of the antagonism by atropine (□), imipramine methiodide (Δ) and desipramine (●) of the carbacholine chloride induced reduction of the release of ³H NA evoked by high K (117) low Na (26 mM) in the rat vas deferens in vitro. Each value is the mean ± S E. (vertical bars) of 4 determinations.

The effect of carbacholine chloride was antagonized by atropine (IC_{50} = 6 10^{-8} M) and by imipramine methiodide (IC_{50} 2 10^{-6} M) but desipramine had poor antagonizing (Fig. 7)

The release evoked by high (hypertonic) K (75 mM) was not antagonized by carbacholine chloride neither at 10 nor at 5 min's incubation. The release evoked by veratridine was slightly (17%) antagonized by carbacholine (Table V). Atropine counteracted carbacho-

Table III. Effect of bethanidine on the release of ³H NA evoked by various means. The concentration of bethanidine was 2.5 10^{-5} M. Each value is the mean ± S.E. of 4 determinations.

Releasing agent	Δ Release, % ± S E.			
	Normal tissue		Reserpinized tissue	
	Control	Bethanidine	Control	Bethanidine
(+)-Amphetamine, 5 10^{-4} M	7.6 0 9	9 8 ± 1.9	31.0 ± 1 6	30 0 ± 1.3
Veratridine 5 10^{-4} M	32.7 ± 0 9	1 8 ± 0 7***	34 6 ± 1 7	0 ± 0 4
Low Na 26 mM	7.6 ± 1.2	8 9 ± 0 1	15.2 ± 1.9	17.5 ± 2.2
High K 75 mM	4 0 ± 0 4	6 6 ± 1.5	14.3 ± 1 7	8 6 ± 1 0
117 mM K 26 mM Na	37 5 ± 0 9	33 6 ± 0 7*	56 7 ± 1 7	54 9 ± 0.9

* P < 0.05 *** P < 0.001 (t-test).

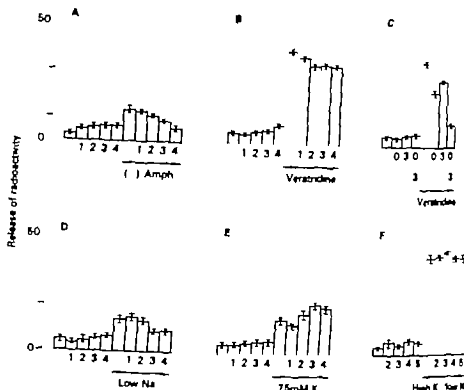


Fig. 5. Antagonism by imipramine methiodide of the release of H NA from normal rat vas def evoked by arious means. The concentrations of the compounds were the same as in the exper shown in Fig. 4

Effect of carbacholine chloride Dubey *et al* (1975) have shown that stimulation of musc nic receptors on noradrenergic nerve terminals reduces the release of NA produced high external concentration of K We therefore examined, if the release of ^{3}H-NA fr the vas deferens evoked by the various means examined was influenced by muscari agonists. In the non reserpinized tissue the release by high K + low Na was reduced w maximally 56% by carbacholine chloride (Table V) and metacholine chloride. The co centration of these agents producing half of this maximal response was 5.5 10^{-3} M f carbacholine chloride and 3 1 10^{-4} M for metacholine chloride.

TABLE II. Effective concentrations of imipramine methiodide and desipramine in antagonizing the rel of H NA I normal nd reserpinized rat vas deferens *in vitro* The alues are interpolated fr dose response curves of the type shown in Fig. 4 and 5.

	EC_{50}, µM			
	Imipramine methiodide		Desipramine	
	Control	Reserpine	Control	Reserpine
(+)-Amphetamine 5 10^{-6} M	11 8	16.2	0.10	0.50
Low Na (26 mM)	14 7	8.8	—	0.35
Veratridine (5 10^{-4} M)	> 245	8.8	18	0.28
High K (117 mM) + low Na$^+$ (26 mM)	> 245	245	80	50

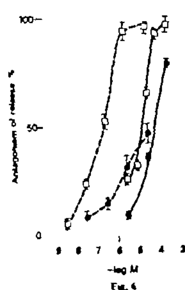


Fig. 6

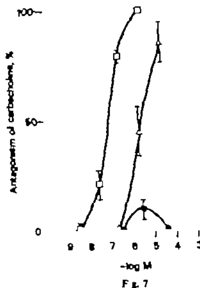


Fig. 7

6 Antagonism by various concentrations of desipramine on the release of H NA evoked by veratridine 10^{-4} M (□) and high K (117 mM) low Na (26 mM) (●) in nontreated (whole lines) and reserpinized (bed lines) rat vas deferens. The incubation time as 20 min in the eratridine experiments nd 10 min hose with high K low Na^+ Each value is the mean ± S E. (vertical bars) of 4 determinations.

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BLE III Effect of bethoxycaine on the release of H-NA evoked by various means. The concentration of bethoxycaine as 2 9 10^{-4} M. Each value is the mean ± S E. of 4 determinations.

	Δ Release, ± S E.			
	Normal tissue		Reserpinized tissue	
	Control	Bethoxycaine	Control	Bethoxycaine
) Amphetamine 5 10^{-5} M	7 6 ± 0 9	9.8 ± 1 9	31.0 _ 1.6	30 0 ± 1.3
atridine, 5 10^{-5} M	32.7 _ 0 9	1.8 _ 0 7*	34 6 ± 1.7	0 ± 0 4***
w Na 26 mM	7 6 _ 1 2	8 9 ± 0 3	15.2 ± 1 9	17.5 ± 2.2
gh K 75 mM	4 0 0 4	6 6 _ 1.5	14 3 ± 1 7	8 6 ± 1 0
17 mM K 26 mM Na	37 5 _ 0 9	33.6 0 7*	54 7 + 1 7	54 9 ± 0 9

p 0.01, *** p 0 001 (t-test).

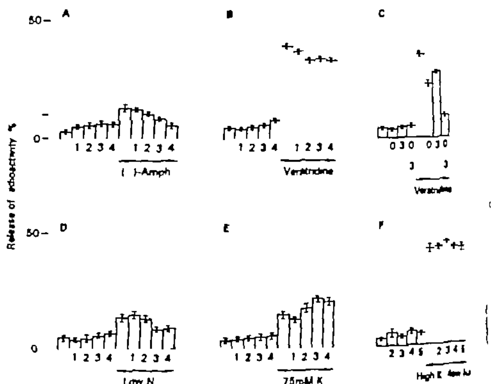
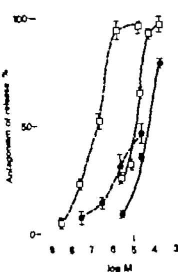


Fig. 5 Antagonism by imipramine methiodide of the release of ³H NA from normal rat vas deferens evoked by various means. The concentrations of the compounds were the same as in the experiment shown in Fig. 4.

Effect of carbacholine chloride Dubey *et al* (1975) have shown that stimulation of muscarinic receptors on noradrenergic nerve terminals reduces the release of NA produced by high external concentration of K We therefore examined if the release of ³H-NA from the vas deferens evoked by the various means examined was influenced by muscarinic agonists. In the non reserpinized tissue the release by high K + low Na was reduced with maximally 56% by carbacholine chloride (Table V) and metacholine chloride. The concentration of these agents producing half of this maximal response was 5.5 10⁻⁸ M for carbacholine chloride and 3 1 10⁻⁸ M for metacholine chloride.

TABLE II Effective concentrations of imipramine methiodide and desipramine in antagonizing the release of ³H NA in normal and reserpinized rat vas deferens *in vitro*. The values were interpolated from dose response curves of the type shown in Fig. 4 and 5

	EC_{50} µM			
	Imipramine methiodide		Desipramine	
	Control	Reserpine	Control	Reserpine
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Low Na (26 mM)	14 7	8.8	—	0.35
Veratridine (5 10⁻⁵ M)	> 245	8.8	18	0.28
High K⁺ (117 mM) + low Na⁺ (26 mM)	> 245	245	80	50



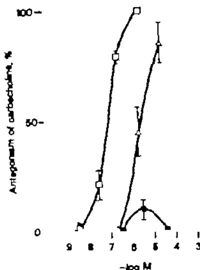


Fig. 6. Antagonism by various concentrations of desipramine on the release of ³H-NA evoked by veratridine 10^{-4} M (□) and high K (117 mM) low Na (26 mM) (●) in nontreated (whole lines) and reserpinized (dashed lines) rat vas deferens. The incubation time was 20 min in the veratridine experiments and 10 min in those with high K + low Na⁺. Each value is the mean ± S.E. (vertical bars) of 4 determinations.

Fig. 7. Dose response curves of the antagonism by atropine (□), imipramine methiodide (Δ) and desipramine (●) of the carbacholine chloride induced reduction of the release of ³H-NA evoked by high K (117 mM) low Na (26 mM) in the rat vas deferens *in vitro*. Each value is the mean ± S.E. (vertical bars) of 4 determinations.

The effect of carbacholine chloride was antagonized by atropine (IC_{50} = 6 10^{-8} M) and by imipramine methiodide (IC_{50} 2 10^{-6} M) but desipramine had poor antagonizing action (Fig. 7).

The release evoked by high (hypertonic) K (75 mM) was not antagonized by carbacholine chloride neither at 10 nor at 5 min s incubation. The release evoked by veratridine was only slightly (17 %) antagonized by carbacholine (Table V). Atropine counteracted carbacho-

Table III. Effect of bethoxycaine on the release of ³H-NA evoked by various means. The concentration of bethoxycaine was 2.9 10^{-4} M. Each value is the mean ± S.E. of 4 determinations.

Releasing agent	Δ Release ± S.E.			
	Normal tissue		Reserpinized tissue	
	Control	Bethoxycaine	Control	Bethoxycaine
(+)-Amphetamine, 5 10^{-4} M	7.6±0.9	9.8±1.9	31.0±1.6	30.0±1.3
Veratridine, 5 10^{-4} M	32.7±0.9	1.8±0.7***	34.6±1.7	0±0.4**
Low Na 26 mM	7.6±1.2	8.9±0.3	15.2±1.9	17.5±2.2
High K 75 mM	4.0±0.4	6.6±1.5	14.3±1.7	8.6±1.0
117 mM K 26 mM Na	37.5±0.9	33.6±0.7*	56.7±1.7	54.9±0.9

* P 0.05, *** P 0.001 (t-test).

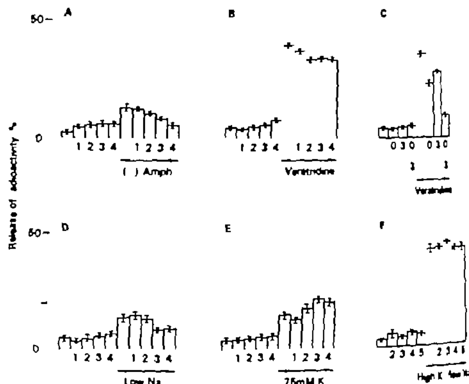


Fig. 5 Antagonism by imipramine methiodide of the release of H NA from normal rat vas deferens evoked by various means. The concentrations of the compounds were the same as in the experiments shown in Fig. 4.

Effect of carbacholine chloride Dubey *et al* (1975) have shown that stimulation of muscarinic receptors on noradrenergic nerve terminals reduces the release of NA produced by high external concentration of K. We therefore examined if the release of ³H-NA from the vas deferens evoked by the various means examined was influenced by muscarinic agonists In the non-reserpinized tissue the release by high K + low Na was reduced by maximally 56% by carbacholine chloride (Table V) and metacholine chloride. The concentration of these agents producing half of this maximal response was 5.5 10^{-8} M for carbacholine chloride and 3 1 10^{-8} M for metacholine chloride.

TABLE II Effective concentrations of imipramine methiodide and desipramine in antagonizing the release of H NA in normal and reserpinized rat vas deferens *in vitro* The values are interpolated from dose response curves of the type shown in Fig. 4 and 5.

	EC_{50}, μM			
	Imipramine methiodide		Desipramine	
	Control	Reserpine	Control	Reserpine
(+)-Amphetamine 5×10^{-6} M	11.8	16.2	0.10	0.30
Low Na (26 mM)	14.7	8.8	—	0.35
Veratridine (5×10^{-5} M)	> 245	8.8	18	0.28
High K⁺ (117 mM) + low Na⁺ (26 mM)	> 245	> 245	80	50

…I. Effect of clonidine, phentolamine and morphine on the release of ³H NA evoked by high K at rat vas deferens. The release of ³H-NA during 10 min was determined. Each value is the mean ± S.E. of 4 determinations.

Compound	Conc. M	Δ Release of ³H-NA, % ± S.E. High K (75 mM)	High K (117 mM) + low Na (26 mM)
—		6.8 ± 0.7	37.7 ± 0.9
Clonidine	3.7 10[illegible]	7.3 ± 1.2	38.5 ± 1.0
Clonidine	3.7 10[illegible]	7.4 ± 0.5	40.2 ± 2.4
—		7.6 ± 1.0	37.0 ± 1.0
Phentolamine	1 10[illegible]	8.5 ± 0.8	35.1 ± 0.7
Morphine	1 10[illegible]	—	38.7 ± 1.4

Discussion

…results obtained are in accordance with the view that the release of NA from peripheral …adrenergic nerve terminals occurs by two mechanisms.

A Ca^{++} dependent release which is evoked by depolarizing agents (high K concentra… and veratridine). This release is probably identical with the exocytotic release of NA (…kins 1968, Smith and Winkler 1977).

Ca^{++} independent, temperature sensitive release evoked by low external Na, (+)-…hetamine and veratridine. Since this release is inhibited by inhibitors of the NA uptake … probably a carrier-mediated transport of NA (Paton 1973, Ross and Kelder 1976 b). … release is easily demonstrated in reserpinized tissue in which the exocytotic release of … is abolished. However, the release induced by (+)-amphetamine, low external Na … a part of that evoked by veratridine obviously occurs with this mechanism also in normal …e.

…he exocytotic release evoked by high K concentration has several similarities with the …lse propagated release of NA (Thoa et al. 1975). Thus, the muscarinic receptor agonistic … carbachol counteracted the release evoked by high K + low Na, which is in …rd with the hypothesis that muscarinic receptors on the NA nerve terminals modulate … release of NA probably by antagonizing the influx of Ca^{++} induced by the depolariza…n of the nerve membranes (Dubey et al. 1975). Like atropine imipramine methiodide but … desipramine antagonized the effect of carbachol which indicates that imipramine …hiodide has an anticholinergic effect.

The potent pre-junctional α-adrenoceptor stimulating agent clonidine antagonizes and … α-blocking compound phentolamine enhances the impulse propagated release of ³H NA (…arke 1977). However, in our system these compounds did not influence the K evoked …ase of ³H-NA in normal vas deferens. This observation agrees with those recently re…rted by Wakade and Wakade (1977) and by Garcia et al. (1978) in slices of the cat spleen. …ese latter authors suggested that the adrenoceptors on the nerve terminals are not able … modulate the release of NA when the membranes are strongly and long-lastingly de…larized by high K concentrations.

In accordance with the studies of Wakade and Kirpekar (1974) and Paton and Lauzon (…77) we found that the release of ³H-NA evoked by high K concentrations in the reser…

TABLE IV Ca²⁺ dependence of the release of H NA evoked by various means. The Ca²⁺-free medium contained 1 mM EGTA. The radioactivity released into the medium during the was expressed in per cent of the radioactivity in the tissue at the start of the release period. Each value is the mean ± S.E. of 4 determinations.

Releasing agent	+ Δ Release % ± S.E.			
	Normal tissue		Reserpinized tissue[a]	
	Control	− Ca²⁺	Control	Ca²⁺
(+)-Amphetamine 5 10^{-4} M	9 1 ± 0.7	9 0 ± 0.7	32.3 ± 1 4	32 6 + 4 0
Low Na 26 mM	9.8 ± 0 4	8.8 ± 0.8	21 1 ± 2.0	18 4 ± 1 0
High K 75 mM	6.8 ± 1.5	1 0 ± 0.5	8.0 ± 2.2	13 3 ± 1 9
117 mM K⁺ + 26 mM Na	38.1 ± 1.3	14.5 ± 0.3	56.3 ± 1.8	44 7 ± 4 2**

$p < 0.01$ $p < 0.001$ (t-test).
Reserpine 5 mg/kg i p. 18 hours before the experiments.

line also in this respect The release produced by (+)-amphetamine or by low external Na⁺ was not influenced by carbacholine (Table V)

In the reserpinized tissue the release induced by high K + low Na was slightly but significantly reduced by carbacholine chloride (Table V) This effect of carbachol was antagonized by atropine and imipramine methiodide. The release evoked by the other means was not influenced by carbacholine in the reserpinized tissue.

Effect of α-adrenoceptor stimulating and blocking agents The pre-junctional α-adrenoceptor stimulating compound clonidine and the α-blocking agent phentolamine failed to influence the release of H NA by high K + low Na or by hypertonic K (75 mM) in the rat vas deferens (Table VI)

Morphine At the concentrations examined morphine did not change the release of ³H-NA induced by high K + low Na (Table VI)

TABLE V Effect of carbacholine chloride (5 5 10^{-4} M) on the release of H NA in rat as deferens evoked by various means. The release f H NA during 10 min was determined. Each alue is the mean ± S.E. of 4 determinations.

	+ Δ Release of H NA, % ± S.E.			
	Normal tissue		Reserpinized tissue	
	Control	Carbacholine	Control	Carbacholine
(+)-Amphetamine, 5 10^{-4} M	6 2 ± 1 9	11 0 ± 1 1	31 0 ± 1 6	29 6 + 2.7
Low Na (26 mM)	9 0 ± 1 0	8 8 ± 1 4	15.2 ± 1 9	11.6 ± 0 8
High K (117 mM) + low Na (26 mM)	35.2 ± 0.6	18.2 ± 0.8	49.6 ± 3.3	38 9 ± 3 4
Veratridine 5 10^{-4} M	32.5 ± 1 4	27 1 ± 0 9	32.5 ± 1 4	33.3 ± 1 9

$p < 0.01$ (t-test).

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pinized tissue is mainly independent on the presence of Ca^{++} and is accordingly not antagonized by muscarinic agents The slight reduction of the release evoked by high K low Na^+ observed when Ca^{++} was omitted may be due to a rest of vesicular ^{3}H-NA release by exocytosis. The release by high K concentrations differed also from that induced by the other means examined in that it was rather resistant against uptake inhibitors. Thus, times higher concentrations of desipramine was necessary to antagonize this release, at the same concentrations which caused membrane stabilizing effect in the vesicular experiment in normal tissue However it seems unlikely that the antagonism was produced by this mechanism, since bethoxycaine had no effect in membrane stabilizing concentrations. A possible explanation is that the release by high K in the reserpinized vasa occurs by the same carrier mediated mechanism as that evoked by the other agents but that the affinity of the uptake inhibitors are strongly reduced by high K^+ concentrations.

The biphasic dose response curve of (+)-amphetamine with an optimal releasing effect at about 1 10^{-4} M is similar to that previously observed for the release of ^{3}H-bretylium in the rat vas deferens (Ross and Kelder 1976 a, 1977) and for the release of ^{3}H-NA in the rat heart atrium (Ross and Renyi 1978). The reason for the decrease at high concentrations of (+)-amphetamine is unclear We have previously suggested the (+)-amphetamine at these concentrations inhibits the membrane transport mechanism (Ross and Kelder 1977) but might alternatively be due to other unidentified membrane effects.

It can be questioned as to whether the carrier-mediated release of NA has any physiological or pharmacological importance under *in vivo* conditions. It seems at least possible that the effect of indirectly acting sympathomimetic amines occurs by this release mechanism, although it is yet unclear if the uptake inhibitors block the uptake of these amines into the nerve terminals or antagonize the carrier-mediated release of the transmitter (Ross 1976). At the depolarized state of the neuronal membranes during nerve activity the carrier-mediated release is probably of little if any importance, since the concentration of the amine in the neuroplasma is normally low However it appears likely that the re-uptake of the transmitter is diminished or abolished when the membranes are depolarized. At high stimulation frequency this type of release may become significant

The present study shows that the release of H NA in reserpinized vas deferens is very similar to that of H bretylium in the normal (non-reserpinized) vas deferens (Ross and Kelder 1976 a, b) 1977) It is therefore likely that H bretylium utilizes the NA membrane transport mechanism. Since bretylium is not released by the Ca dependent exocytotic release and passes the neuronal membrane by passive diffusion much slower than does NA and is not isotopically diluted in the tissue H bretylium appears to be a good model compound in studies of the membranal NA transport system.

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stigate whether membrane fusions and histamine release occur when isolated intact cell granules, surrounded by normal perigranular membranes, are incubated in Ca^{++} ning solutions.

Methods

ration of intact mast cell granules

le Sprague-Dawley rats (350–450 g) were used in each of the 12 expts. performed. The rats were anaesthetized under light ether anaesthesia by cutting the carotids and a small incision was made along the ne of the abdomen. The peritoneal cavity was washed with 9 ml of salt solution (NaCl 145 mM, KCl M, $CaCl_2$ 0.9 mM) buffered with 10 v/v Sörensen phosphate buffer (Na_2HPO_4 + KH_2PO_4, 67 mM), to which 1 mg of human serum albumin/ml was added. The pleural cavity was washed in an ous fashion. The mixed cell suspension was layered onto a discontinuous 30–40% Ficoll gradient. radients were centrifuged (350 g, 10 min) and the mast cells were collected from the diffuse band interface between the 30 and 40 Ficoll layers and from the bottom of the 40 Ficoll layer. The cells were washed twice in the above buffered salt solution containing human serum albumin. By this od, originally developed by Uvnäs and Thon (1959), a cell suspension was obtained containing more 95% mast cells.

Mast cells were suspended in 3.1 ml ice-cold 0.34 M sucrose buffered with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), a tissue culture buffer without metal binding capacity (Good 1969; Edwards *et al.* 1974). The pH was adjusted to 7.0 with 1.0 N NaOH (Na concentration 5 mM). 3 ml aliquots cooled in ice were sonicated in each of the 12 expts. with a MSE 100 W ultrasonic disintegrator set at 4 µm amplitude for 5 using a probe with a diameter of 10 mm. The sonicated suspensions were centrifuged (350 g, 15 min, 4°C) and the granule-containing supernatants, containing than 60 "intact" granules, were then centrifuged (3 000 g, 20 min, 4°C).

ation procedure

granule-containing pellets were incubated for 45 min at 25°C in 240 µl 0.34 M sucrose buffered with 5 mM HEPES, pH 7.0. To this incubation medium was added either $CaCl_2$ (10 mM, 1 mM, 100 µM, µM), $MgCl_2$ (10 mM) or NaCl (10 mM) as control. In another 4 expts. lower concentrations of $MgCl_2$ µM, 100 µM, 10 µM) were used.

chemical analysis and electron microscopy

After completing the incubation procedure half of each granule-containing suspension was taken for histamine (Shore *et al.* 1959, as modified by Bergendorff and Uvnäs 1972) and protein (Lowry *et al.* 1951) ... after previous centrifugation and washing in 0.15 M NaCl buffered with Sörensen phosphate buffer (Na_2HPO_4 + KH_2PO_4, 67 mM), pH 7.0, to release any histamine remaining in the "changed" granules. histamine in the granule-containing suspension is then located solely in intact rat mast cell granules surrounded by a perigranular membrane. The other half of the granule suspension (120 µl) was processed for electron microscopy. The granules were fixed with 60 µl 10 glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, for 20 min at 22°C. The suspension was then cooled down in an ice-bath for 5–10 min after adding 300 µl ice-cold 1.5 OsO_4 in 0.1 M cacodylate buffer. After fixation and centrifugation for 25 min, the fixation was continued in new 1.5 OsO_4 in the same buffer (35 min). The granule obtained after centrifugation and washing twice in 0.1 M cacodylate buffer, pH 5.0, was stained ... in 0.5 uranyl acetate (pH 5.0, 22°C, 30 min). After washing in 0.1 M acetate buffer, pH 5.0, granule pellet was suspended in freshly prepared 2 agar (60°C) before being dehydrated in an ... series, soaked in propylene oxide and finally embedded in Spurr low-viscosity embedding media. ... sections for electron microscopy were cut on a Reichert ultramicrotome Om U3 and stained with ... uranyl acetate and diluted lead citrate before examination in a Philips EM 301 electron microscope at 80 kV. ... thin sections for light microscopy were stained with a solution of toluidine blue and azur A containing ... sucrose.

Materials

Human serum albumin (AB Kabi, Stockholm, Sweden), Ficoll (AB Pharmacia, Uppsala, Sweden), HEPES ((hydroxyethyl)-1-piperazine-ethanesulfonic acid, Calbiochem, Los Angeles, California, USA), glutaraldehyde 25 — especially purified for electron microscopy and Spurr low-viscosity embedding media (AB Laboratories, Reading, England) were used. All other substances were obtained from the usual ... sources.

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The influence of calcium on morphology and histamine content of isolated intact rat mast cell granules

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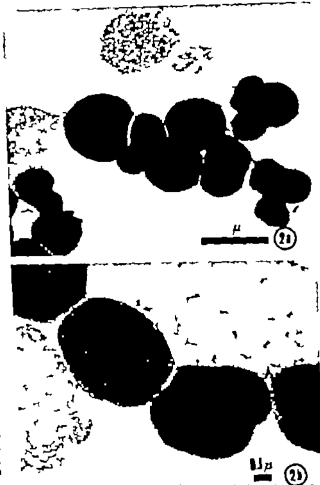
Abstract

ANDERSON P., P RÖHLICH and B UVNÄS. *The influence of calcium on morphology and histamine content of isolated intact rat mast cell granules.* Acta physiol. scand 1979 105 350–358

Histamine is released by "sequential exocytosis" in mast cells. The exocytosis involves fusion of the plasma membrane with the perigranular membrane and further fusions of adjacent perigranular membranes. To study a possible direct effect of Ca^{++} on granule membrane fusions, mast cell granule suspensions were prepared from sonicated rat mast cells. With the sonication method used, more than 60 % of the granules obtained were found to be homogeneous, electron dense and surrounded by a perigranular membrane, as observed in the electron microscope. These granules correspond to normal, histamine-containing granules found in untreated mast cells and are therefore named "intact" granules. The other granules were swollen, less electron dense and without a perigranular membrane. These "changed" granules are formed during the histamine release process. Aliquots of the granule suspension were incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, containing different concentrations of $CaCl_2$, $MgCl_2$ (10 mM, 1 mM, [illegible] 10 μM) or NaCl (10 mM). Only with the highest concentration (10 mM) of Ca^{++} or Mg^{++} was it possible to visualize an apposition of the perigranular membranes of "intact" normal granules. No [illegible] the individual membrane structures could be observed at the place of membrane contact. Thus, [illegible] no signs of membrane fusions. The histamine content was lower in the suspensions incubated with low concentrations of these ions or with 10 mM NaCl. Ca^{++} and Mg^{++} in high concentrations seemed to stabilize the perigranular membranes instead of initiating histamine release. Therefore, changes in the Ca^{++}-ion concentration per se do not explain the membrane fusions seen in mast cells during "sequential exocytosis".

When sensitized mast cells are challenged with antigen (Anderson *et al.* 1973) or when normal mast cells are treated with, for example, compound 48/80 (Röhlich *et al.* 1971), histamine is released by sequential exocytosis. Sequential exocytosis (Röhlich *et al.* 1971) involves fusion of the plasma membrane with the perigranular membrane and further fusions between adjacent perigranular membranes. The granules involved in the [illegible] process alter from normal homogeneous, electron-dense granules to "changed" granules with a swollen, less electron-dense, reticular net like appearance in the electron microscope.

Since the histamine release process is known to be Ca^{++}-dependent (Mongar and Schild 1958, Mota 1959 Mota and Ischii 1960, Chakravarty 1960, Högberg and Uvnäs 1960, Foreman and Mongar 1972, Douglas and Ueda 1973) it was considered of interest



2. Mast cell granules incubated in 0.34 M sucrose buffered ... 10 mM HEPES, pH 7.0, ... 10 mM $CaCl_2$. The ... granules (with perigranular membranes) formed either aggre... or long chains of granules. ... fication 2 ... 17 000 ... 2 b ...

...parable planes of sectioning (Fig. 3 a). The adhering membranes often also showed a ...ve-like pattern (Fig. 3 b). At the highest magnifications used, a 5-layered structure was ...er ed, ... here the membranes were in contact (Fig. 3 a). A fusion of the membranes, ... h an elimination of membrane layers from this pentalaminar appearance of the membrane contact, yielding a trilaminar diaphragm, or further elimination of individual layers ...ulting in a single-layered diaphragm (pore), was never observed in our material, when ... mM $CaCl_2$ was used in the incubation medium.

The mixed granule suspensions were also incubated in 10 mM MgCl... in 0.34 M sucrose ...ffered with 10 mM HEPES, pH 7.0. The ultrastructural appearance was similar to that ...nd ... hen 10 mM $CaCl_2$ was used. The intact granules again formed aggregates, often in ...e form of long chains. The perigranular membranes in contact either showed a wave-like ...ttern or were parallel to each other for a long distance, forming a 5-layered substructure ...ig. 3 c). Fusion of the membranes did not occur



Fig. 1 Mast cell granules incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, [illegible] 10 mM NaCl (a) Homogeneous, electron-dense "intact" "normal" granules and swollen, less electron-[illegible] "changed" granules are seen. No contact between the granules is observed. (b) Two intact granules [illegible] to each other A perigranular membrane can be seen around each of the granules. The [illegible] membranes are not in contact with each other (Magnification. 1 a 14 000 × 1 b 57 500)

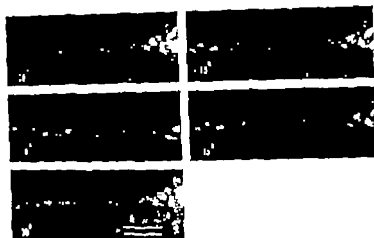
Results

Electron microscopy

The mast cell granule suspensions, obtained by our technique using ultrasonic disintegration of mast cells and differential centrifugations, were uncontaminated by other cell organelles. Between 60 and 70 % of the granules had a normal appearance (intact granules). They were surrounded by a perigranular membrane. These granules were homogeneous, electron-dense and thus similar to those found in untreated mast cells. The other granule type was less electron-dense with a swollen, reticular net-like appearance and thus corresponds to the "changed" granules found in mast cells during the histamine release process.

When mast cell granules were incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0 containing 10 mM NaCl (control medium), the granules were seen to have the same appearance as described above (Fig. 1 a) and in earlier experiments using a medium consisting of only 0.34 M sucrose (Anderson *et al* 1974) At these magnifications it was evident that the granules were lying separately and not in contact with each other (Fig. 1 a, b) The electron dense granules were surrounded by a membrane (Fig. 1 b), which was missing around the "changed" granules.

A completely different picture was observed when the mast cell granule suspensions were incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, containing [illegible] CaCl (Fig. 2 and 3) Most of the normal intact granules were adhering to similar homogeneous granules, forming aggregates or long chains of granules (Fig. 2 a, b). The adherent membranes often maximized their area of contact, leading to distortions of the unmodified spherical granules. A "changed" granule could sometimes also be associated with the aggregates of granules. At higher magnifications it was observed that the membranes [illegible] contact were often oriented parallel to each other (Fig. 3 a) and adhering to each other [illegible] the contact site. The trilaminar structure of the individual , could be observed [illegible]



4 Mast cell granules incubated as in Fig. 2. By tilting the specimen in goniometer stage the con-
ty of the separate membranes can be seen (±0°) (Magnification 102 000)

bated in 0.34 M sucrose buffered with 10 mM HEPES (pH 7.0) containing 10 mM $CaCl_2$
0 mM $MgCl_2$ was higher (0.127 and 0.110 respectively) than in the granule suspensions
bated with 10 mM NaCl (0.089). *The difference is statistically significant at the 1 %*
l. When the granule suspensions were incubated in lower concentrations of $CaCl_2$
, $MgCl_2$), on the other hand, the histamine content did not differ from the control
ubations.

Discussion

exocytosis, the secretory products stored in membrane-limited vesicles or granules are re
sed to the extracellular space without preliminary discharge into the cytoplasm and
hout concomitant loss of cytoplasmic constituents. It has been shown that an
racellular increase of calcium ions is essential for exocytosis in many cell types. However
s not known how Ca induces the fusion process of exocytosis.

As we have shown earlier (Rohlich *et al.* 1971 Anderson *et al* 1973 Anderson 1975),
st cells are an excellent model for studying exocytosis. In mast cells the exocytosis is

LE 1. The effect of Ca^{2+} and Mg^{2+} on the histamine content (histamine/protein ratio) of isolated intact mast cell granules

	Mean	S D
[illegible] M $CaCl_2$	0.127	0.051
[illegible] mM $CaCl_2$	0.094	0.034
[illegible] μM $CaCl_2$	0.067	0.040
[illegible] μM $CaCl_2$	0.086	0.043
[illegible] mM $MgCl_2$	0.110	0.049
[illegible] M NaCl	0.089	0.030

P 0.01

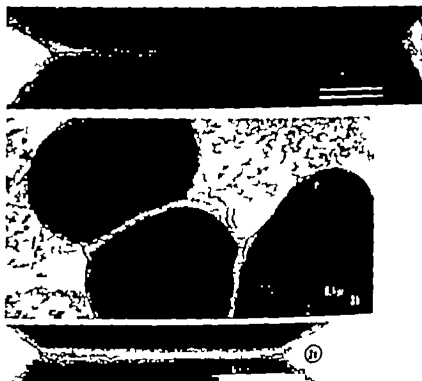


Fig. 3 Mast cell granules were incubated as in Fig. 2. (a) The perigranular membranes are oriented parallel to each other at the contact site. The trilaminar structure of each membrane can be observed at this high magnification. (b) The opposed membranes often also showed a ... pattern. (c) Mast cell granules incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, containing 10 mM $MgCl_2$. The layered structure is easily seen, where the membranes oppose each other. No fusion of the membranes occurs. (Magnification 3 a 165 000 3 b 48 000 3 c 165 000)

Obliquely sectioned membranes often gave the impression of a "fusion" of the adjacent membranes. To exclude the possibility of an incorrect interpretation, we tilted some of our specimens in a goniometer stage of the electron microscope. In all of such tilted specimens we were able to follow the continuity of the separate membranes and exclude a real fusion between the membranes in contact (Fig. 4).

When the mixed mast cell granules were incubated with 0.34 M sucrose buffered with 10 mM HEPES pH 7.0 containing lower concentrations of CaCl (1 mM, 100 μM, 10 μM) or MgCl (1 mM 100 μM 10 μM) the same morphology was found as in the control suspensions containing 10 mM NaCl in HEPES-buffered sucrose.

Histamine content

Histamine and protein were analyzed in the granule fractions after completing the incubations in 0.34 M sucrose buffered with 10 mM HEPES pH 7.0 containing different concentrations of $CaCl_2$, MgCl or NaCl. The histamine contents of the fractions were expressed as the histamine/protein ratio (Table 1). This ratio also reflects the proportion of "intact" granules in the fractions, since only "intact" granules surrounded by a perigranular membrane retain histamine, when washed in isotonic sodium chloride solution (Uvnäs *et al.* 1970 Anderson *et al.* 1974). The histamine granule fraction

hould be cautious when interpreting electron micrographs of subcellular fractions, as branes may become more fragile during in vitro preparation procedures, leading to ctural membrane ruptures.

ocytosis in mast cells is dependent not only on Ca^{++} but also on energy (Hogberg and is 1957 Peterson 1974). It is not yet known, however where energy is utilized during dease process. Therefore it is conceivable that the intracellular increase in the calcium entration is just the initial step in a chain of events leading to complete fusion of the branes. After stimulation of mast cells, calcium may thus act as a secondary mes- er and activate some mechanism that requires metabolic energy If this is the case, fusions would be expected in an in vitro system. A candidate for such a calcium and gy-requiring system could be a contractile acto-myosin system (microfilaments), the ficance of which in exocytosis is still debated (Röhlich 1975).

he adhesive property of calcium and magnesium ions in our experiments is probably specific for the release process, but may be explained by the divalent character of the Thus, it is known that these divalent ions play an important role in adhesions of branes and cells in general. The adhesive effect can probably be explained by the fact the ions act as bivalent bridges between negatively charged groups on the membrane aces (cf Banks 1966). Our results showed a significantly higher histamine content in ule preparations treated with high Ca^{++} or Mg^{++} concentrations (10 mM). This finding be due to a stabilizing effect of these ions on the perigranular membrane, instead of ucing a fusion process between them.

investigation has been supported by grants from The Swedish Medical Research Council (B 77-04X 3A and B 78-04X 39-14B) and Jeansson s, Wiberg and Bergvall Foundations.
e skilled technical assistance of Miss Ann Nilsson is grateful acknowledged.

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sequential in space and time ("sequential exocytosis"). This means that not all the gran-
move to the plasma membrane. Instead there is a chain-like fusion of the membrane-limited
granules Sequential exocytosis in mast cells has been reported by other authors, to
(Lagunoff 1972, 1973 Douglas 1974 a b)

As in other cell types, Ca^{+} is necessary for the secretory process in mast cells (for referen
see Introduction) However it is still unclear how calcium is involved in membr
fusions during exocytosis. One of the possibilities is a direct calcium effect on the me-
branes, leading to fusions of adjacent perigranular membranes.

To investigate the possibility of a direct effect of calcium on the membranes we studi
a pure mast cell granule fraction consisting of a high percentage of normal, ma
membrane limited granules These suspensions were assumed to be a suitable model fo
studying membrane fusions, since the perigranular membrane is able to fuse during ex
exocytosis not only with plasma membranes but also with other perigranular membrane

As seen from the results, only when using the highest concentration of $CaCl_2$ (10 m
we were able to observe an effect of calcium on the perigranular membranes. Instead of
fusion, there was a close apposition of perigranular membranes, resulting in a pentalam
appearance of the membranes in contact Even at places where it was difficult to disc
the individual membranes in contact and which could be taken as a place of fusion, we co
show by tilting the specimens in a goniometer stage, that the apposed membranes w
intact and distinct This apposition of the membranes does not seem to be specific f
calcium, since magnesium in the same concentration had a similar effect. In fact, it h
been shown that magnesium was not effective in inducing exocytosis when injected in
mast cells (Kanno *et al* 1973). In addition, the calcium concentration which induc
adhesion of perigranular membranes in our experiments is probably well above the equi
intracellular calcium level during stimulation in other cell types.

Experiments on the effects of calcium on isolated secretory vesicles from other cell ty
are contradictory Edwards *et al* (1974) described apposition and rupture of perigran
membranes in isolated chromaffin granules suspended in high concentrations of calc
or magnesium (5–10 mM) Similarly Dahl and Gratzl (1976) observed calcium-induc
fusions of isolated secretory vesicles from the islets of Langerhans. Using fre
fracturing to detect membrane fusions, they found the effective calcium concentration
be much lower (*i.e* 1 μM).

On the other hand, Schober and collaborators (1977) found an apposition and a penta
minar structure but no fusions at the places of membrane contact in isolated chroma
granule fractions, when using 10 mM $CaCl_2$. Palade was also unsuccessful in induc
in vitro fusion by calcium ions of membrane limited granules isolated from pancre
acinar cells (personal communication 1976).

These contradictory results have various explanations. The experiments cited ab
differed from each other in biological material, isolation procedures, incubation m
and electron microscopic techniques. Some of the prerequisites for exocytotic membr
fusions may be lacking in some of these in vitro systems (energy enzymes, microfilam
etc.) Moreover one can not exclude the possibility that specific sites on the membranes
calcium are altered or completely lost during the preparation procedure. On the other h

Acta physiol. scand. 1979 105. 359–366

From the Department of Pharmacology and the Department of Alcohol and Drug Addiction Research, Karolinska Institutet, Stockholm, Sweden

Determination of 6-keto-prostaglandin $F_{1\alpha}$ in rabbit kidney and urine and its relation to sodium balance

By

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Abstract

Oliw E., I. Lundén, B. Sjöquist and E. Änggård. *Determination of 6-keto-prostaglandin $F_{1\alpha}$ in rabbit kidney and urine and its relation to sodium balance.* Acta physiol. scand. 1979. 105. 359–366.

The occurrence of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) was demonstrated in rabbit kidney medulla and cortex by mass-spectrometry. The post-mortem accumulation of 6-keto-$PGF_{1\alpha}$ was studied by mass-fragmentography in regions of the rabbit kidney using (3,3,4,4-$^2H_4$) 6-keto-$PGF_{1\alpha}$ as an internal standard. The cortex contained 1.4 ± 0.3 µg/g, the medulla 2.1 ± 0.6 µg/g and the papilla 3.7 ± 0.7 (S.E.) µg/g. The amount of 6-keto-$PGF_{1\alpha}$ is thus about 5 fold higher in the cortex than reported for PGE_2 and $PGF_{2\alpha}$. The post-mortem accumulation of PGE_2 and $PGF_{2\alpha}$ dominates over 6-keto-$PGF_{1\alpha}$ in the medulla and the papilla. The occurrence of 6-keto-$PGF_{1\alpha}$ in rabbit urine was demonstrated by mass-fragmentography. On a low salt diet, unanaesthetized, female rabbits (n = 6) excreted Na 0.09 ± 0.01 (S.E.) mmol/day, 6-keto-$PGF_{1\alpha}$ 11.8 ± [illegible] µg/day and immunoreactive $PGF_{2\alpha}$ (i$PGF_{2\alpha}$) 2.0 ± 0.6 µg/day. Two day treatment with acetylsalicylic acid (100 mg/kg × 2) reduced urinary excretion of 6-keto-$PGF_{1\alpha}$ and i$PGF_{2\alpha}$ by 71 and 85 %, respectively (p < 0.05), but sodium excretion was unchanged. On the same diet supplemented with NaCl, the rabbits excreted Na 27.5 ± 3.4 mmol/day (p < 0.05), 6-keto-$PGF_{1\alpha}$ 13.3 ± 4.6 µg/day (p > 0.05) and i$PGF_{2\alpha}$ [illegible] 0.10 µg/day (p < 0.05). 6-keto-$PGF_{1\alpha}$ is a major metabolite of prostacyclin (PGI_2). The occurrence of 6-keto-$PGF_{1\alpha}$ in kidney and urine indicates a considerable synthesis of PGI_2 in the kidney. The data on urinary PG excretion indicate that intrarenal synthesis of PGI_2, in contrast to $PGF_{2\alpha}$, is not influenced by dietary variations in NaCl.

Key words: Acetylsalicylic acid, gas chromatography—mass spectrometry, prostacyclin (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), radioimmunoassay, rabbit kidney, sodium deprivation

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The kidney has a high capacity to synthetize prostaglandins (PGs) (Hamberg 1969). The synthesis is predominately located to the renal medulla, which has a tenfold higher capacity to synthetize PGE_2 and $PGF_{2\alpha}$ than the cortex (Larsson and Änggård 1973, Larsson and Änggård 1976, Pong and Levine 1976). Studies on the renal PGs indicate, however, that they might be of physiological importance in both the renal medulla and in the renal cortex. In the medulla, PGs seem to oppose the action of the antidiuretic hormone (Grantham and Orloff 1968, Anderson et al. 1975). In the cortex, PGs might be of importance for renal

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plates through the silicic acid layer. The references were localized by spraying the sides of the plates ... phosphomol)bdic acid followed by gentle heating. The zone of the chromatogram corresponding ... reference (Rf 0.52) was scraped off and eluted with 5% methanol in ethyl acetat (2 ml 2).

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Kidney and urine samples were run on an LKB 2091 GC MS which was equipped with multiple ion ... A 1 ... OV-17 GLC column (90 cm) was used and operated at 250°C. The energy of the electrons ... 22 eV and the trap current was 100 µA. For the mass fragmentographic analysis the instrument was ... focused on m/e 602/598 (M 31) and 512/508 (M 121), but for routine analysis on the higher ... The method for 6-keto-PGF$_{1\alpha}$ analysis is ... modification of the one described by Pace-Asciak ...

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Results

... spectrum of 6-keto-PGF$_{1\alpha}$

... structure of the 6-keto-PGF$_{1\alpha}$ derivate for GC MS analysis, 6-methoxime-PGF ... methyl ester 9,11,15-tris-trimethylsilyl ether is shown in Fig. 1 top. A mass spectrum of ... 4-$^2H_4$) 6-keto-PGF$_{1\alpha}$ is demonstrated in Fig. 1 A. The deuterated 6-keto-PGF$_{1\alpha}$ ... dard contained less than 1% of the protium form. The characteristic ions of high ... values in the upper mass range were m/e 602 (M 31), 56... (M 71), 512 (M 121) and ... (M 161).

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blood flow distribution and for renin release (Larsson and Änggård 1974, Larsson et al. 1974 Weber et al 1976)

In 1976 Moncada, Vane and coworkers discovered that arterial walls can synthesize a novel PG like product from arachidonic acid It s structure was elucidated and the compound was named prostacyclin PGI_2 (Johnson et al 1976). PGI is unstable in aqueous solution, where it is hydrolysed to 6-keto-PGF Since this is the only known pathway for 6-keto-PGF formation, the level of 6-keto-$PGF_{1\alpha}$ in a tissue might be used to estimate PGI biosynthesis (Gibson 1977)

Pace Asciak and Rangaraj (1977) demonstrated that rat renal homogenates form 6-keto-$PGF_{1\alpha}$ from arachidonic acid The physiological function of PGI or 6-keto-$PGF_{1\alpha}$ in the kidney is unknown Recent studies show that exogenous PGI can influence renal function PGI increases renal blood flow and stimulates renin release *in vitro* and *in vivo* (Gerber et al 1978 a, Whorton et al 1977)

The present study describes the regional distribution of the endogenous formation of 6-keto-$PGF_{1\alpha}$ in the rabbit kidney Since variations in sodium intake influence renal PG synthesis in rats (Tobian and O Donnell 1976) and urinary excretion of PGE_2 and PGF in rabbits (Weber et al 1977 Davila et al 1978), we also studied the urinary excretion of 6-keto-$PGF_{1\alpha}$ in rabbits on low and high salt intake.

Material and methods

Materials

All reagents were of analytical grade $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and (3,3 4,4- H_4) 6-keto-$PGF_{1\alpha}$ were gifts from the Upjohn Co (Kalamazoo, USA) through the courtesy of Dr U Axén. The 6-keto-prostaglandins were dissolved in ethyl acetate containing 5 methanol and stored at −20°C. The deuterated 6-keto-$PGF_{1\alpha}$ contained less than 1 of the protium form according to mass fragmentography and was tested for chemical purity by thin layer chromatography Methoxyamine HCl and BSTFA (N,O-bis(trimethylsilyl) trifluoracetamide) were obtained from Pierce Rockford, USA The thin layer silicic acid plates were obtained from Merck (20 20 cm, 0 25 mm DC Fertig-Platten Kieselgel 60). Acetylsalicylic acid was obtained locally H $PGF_{2\alpha}$ (150 Ci/mmol) for use in a $PGF_{2\alpha}$ radioimmunoassay was obtained from Radiochemical Centre, Amersham, England The $PGF_{2\alpha}$-antibodies were raised in our laboratory and characterized as previously described (Oliw et al 1978). The test diets, one basal diet with 0.5 and low salt diet with 0 03 NaCl but otherwise similar were obtained from Ralston Purina C Richmond, Indiana, USA.

Methods

Kidney analysis M le white albino rabbits (2 2.5 kg) were kept on a standard pellet diet (Astra Ewos, Södertälje Sweden, containing 0 5 N Cl) d water *ad libitum* The rabbits were killed by a blow on the head and exsanguinated. To standardize the procedure the kidneys were removed 5 min after death and then immediately put to ice-cold 0 1 M potassium phosphate buffer pH 7 4 The following steps were rapidly performed at 4°C The cortex, medulla nd papilla of each kidney were macroscopically dissected, weighed (0.4–1.5 g) and chopped into small pieces. The papillas from each pair of kidneys were pooled. 4 volumes of the phosphat buffer and 1.0 μg (3,3 4 4- H_4) 6-keto-$PGF_{1\alpha}$ were added before homogenization in a glass tube with 5 strokes of loose f tting Teflon pestle t 2 500 r p.m. The homogenate was centrifuged for 60 min at 90 000 g (Spinco L2 ultracentrifuge).

Proteins in the supernatant were precipitated by addition of 10 volumes of ethanol. The solution was rapidly filtered and evaporated lmost t dryness. The residue was dissolved in 2 ml water acidified to pH 3 with 0.1 M HCOOH and extracted with ethyl acetate (2 ml 2). After evaporation, the samples were purified by preparative thin layer chromatography (TLC) on silicic acid TLC plates (system: ethyl acetat trimethylpentane:acetic acid wat ~110 17 20:100). Reference 6-keto-$PGF_{1\alpha}$ was put on both sides of the plate. The references were separated from the samples by vertical lines drawn on both

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Material and methods

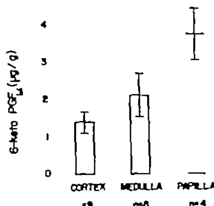
Materials

All reagent were of analytical grade $PGF_{2\alpha}$ 6-keto-$PGF_{1\alpha}$ and (3,3,4,4- H) 6-keto-$PGF_{1\alpha}$ were generous gifts f m the Upjohn Co. (Kalamazoo USA) through the courtesy of Dr U Axén. The 6-keto-prostaglandins were dissolved i ethyl acetate containing 5 methanol and stored at −20°C. The deuterated PGF contained less than 1 of the protium f rm according to mass fragmentography and was checked f r chemical purity by thi layer ch omatography Methoxyamine HCl and BSTFA (N,O-bis-trimethylsilyl) trifluoracetamide) were obtained from Pierce Rockford USA The thin layer silicic acid plates were obtained from Merck (20 20 cm, 0.25 mm DC Fertig-Platten Kieselgel 60). Acetylsalicylic acid was obtained locally H $PGF_{2\alpha}$ (150 Ci/mmol) for use in a $PGF_{2\alpha}$ radioimmunoassay was obtained from Radiochemical Centre Amersham, England. The $PGF_{2\alpha}$-anti bodies were raised in our laboratory and characterized as previously described (Oliw *et al* 1978) The test diets, one basal diet with [illegible] and a low salt diet with 0 03 NaCl but otherwise similar were obtained from Ralston Purina Co [illegible] Indiana, USA.

Methods

Kidney analysis. Male white albino rabbits (2 2.5 kg) were kept on a standard pellet diet (Astra-Ewos Södertälje Sweden, containing 0.5 NaCl) nd water *ad libitum* The rabbits were killed by a blow on the head and exsanguinated. To standardize the procedure the kidneys were removed 5 min after death and then immediately put into ice-cold 0 1 M potassium phosphate buffer pH 7 4 The following steps were rapidly performed at 4°C Th cortex, medulla nd pap lla of each kidney were macroscopically dissected, weighed (0.4–1.5 g) and chopped into small pieces. The papillas from each pair of kidneys were pooled. 4 volumes of the phosphate buffer nd 1 0 μg (3 3 4 4-²H) 6-keto-$PGF_{1\alpha}$ were added before homogenization in a glass tube with 5 strokes of a loose f tting Teflon pestle t 2 500 r p m. The homogenate was centrifuged fo 60 min at 90 000 g (Spinco L2 ultracentrifuge)

Proteins in the supernat nt were precipitated by add tion of 10 olumes f ethanol. The solution was rapidly filtered and evaporated almost t dryness. The residue was dissolved in 2 ml ater acidified to pH 3 with 0.1 M HCOOH and e tracted with ethyl acet te (2 ml 2). After evaporation, the extracts were purified by preparative thin layer chromatography (TLC) on silicic acid TLC plates (ethyl acetat trimethylpentane acetic acid water = 110 17 20:100). R ference 6-keto-$PGF_{1\alpha}$ was put on the sides of the plate The references were separated from the by vertical l nes dra n on the



Post mortem accumulation of 6-keto-PGF$_{1\alpha}$ in of the rabbit kidney. Mean ± S.E.

...e post-mortem accumulation of 6-keto-PGF$_{1\alpha}$ increased from cortex to medulla and ...lla (Fig. 3). The cortex contained 1.4 ± 0.3 µg/g wet weight (n = 9), medulla 2.1 ± 0.6 (n = 8) and the papilla 3.7 ± 0.7 µg/g (n = 4).

...e analysis

... occurrence of 6-keto-PGF$_{1\alpha}$ was demonstrated in rabbit urine by mass fragmento... ...hy which showed the same ratio between different ion pairs, m/e 598/602 and 508/512 ... retention time of the 6-keto-PGF$_{1\alpha}$ derivative (Fig. 2 C).

...he influence of variations in dietary sodium on the urinary excretion of 6-keto-PGF$_{1\alpha}$, ...F$_{2\alpha}$, sodium and potassium in six rabbits is shown in Table I. On the low salt diet the ...ary excretion of Na was 0.09 ± 0.01 mmol/day. The urinary excretion of 6-keto-PGF$_{1\alpha}$ ... 11.8 ± 2.2 µg/day and that of iPGF$_{2\alpha}$ 2.0 ± 0.6 µg/day. Treatment with acetylsalicylic ... (30 mg ... s.c.) for two days reduced 6-keto-PGF$_{1\alpha}$ excretion by 71% to 3.4 ± 1.0 µg/day ... 0.05) and reduced iPGF$_{2\alpha}$ by 85% to 0.3 ± 0.1 µg/day ($p < 0.05$). Sodium and potassium ...retion were unchanged.

...n the high NaCl intake urinary Na excretion was increased 300 fold and potassium ...etion decreased slightly (Table I). Urinary 6-keto-PGF$_{1\alpha}$ excretion was unchanged but ...F$_{2\alpha}$ decreased to 0.63 ± 0.10 µg/day ($p < 0.05$).

...E I. Influence of dietary sodium on urinary excretion of 6-keto-PGF$_{1\alpha}$, iPGF$_{2\alpha}$, sodium and potassium in rabbits (n = 6)

	6-keto-PGF$_{1\alpha}$ (µg/day)	iPGF$_{2\alpha}$[b] (µg/day)	Sodium[b] (mmol/day)	Potassium[b] (mmol/day)
... NaCl intake ...ean ± S.E.)	11.8 ± 2.2	2.0 ± 0.6	0.09 ± 0.01	17.6 ± 0.6
...h NaCl intake ...ean ± S.E.)	13.3 ± 4.6	0.63 ± 0.10	27.5 ± 3.4	12.8 ± 1.6
...ference	NS	p < 0.05	p < 0.05	p < 0.05

...ean of 24 h urinary excretion from 6 rabbits.
...ean of two consecutive 24 h excretions from 6 rabbits.

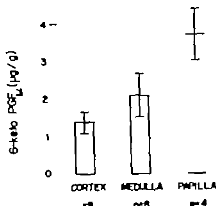
Fig. 1 Top. The structure of the 6-keto-PGF derivate 6-methoxime-$PGF_{1\alpha}$ methyl ester 9,11,15-tri-trimethylsilyl ether which was used for GC MS analysis. A. Mass spectrum of the (3,3,4,4-$^2H_4$)-6-keto-$PGF_{1\alpha}$ standard. B. Mass spectrum of 6-keto-$PGF_{1\alpha}$ from rabbit kidney medulla. Note the difference between the mass spectra of the deuterated standard (A) and the endogenous 6-keto-$PGF_{1\alpha}$ (B) except in the lower mass range

of derivatization for GC MS analysis was therefore always methoxylation of the 6-keto group before methylation of the carboxyl-group (*cf* Pace-Asciak *et al* 1977).

Kidney analysis by mass fragmentography

The specificity of the mass fragmentographic method of 6-keto-$PGF_{1\alpha}$ analysis was demonstrated by identical ratios between two different ion pairs, m/e 598/602 and 508/512 at the retention time of the 6-keto-$PGF_{1\alpha}$ derivate. This is demonstrated by mass fragmentograms from renal cortex and renal medulla (Fig. 2 A and B).

Fig. 2. Mass fragmentograms of 6-keto-$PGF_{1\alpha}$ using the ion pairs m/e 598/602 and 508/512 in (A) rabbit renal medulla, (B) rabbit renal cortex, (C) rabbit urine



Post mortem accumulation of 6-keto-PGF$_{1\alpha}$ in of the rabbit kidney. Mean ± S.E.

e post-mortem accumulation of 6-keto-PGF$_{1\alpha}$ increased from cortex to medulla and la (Fig. 3). The cortex contained 1.4 ± 0.3 µg/g wet weight (n = 9), medulla 2.1 ± 0.6 (n 8) and the papilla 3.7 ± 0.7 µg/g (n = 4).

analysis

occurrence of 6-keto-PGF$_{1\alpha}$ was demonstrated in rabbit urine by mass fragmentography which showed the same ratio between different ion pairs, m/e 598/602 and 508/512 e retention time of the 6-keto-PGF$_{1\alpha}$ derivative (Fig. 2 C).

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E I. Influence of dietary sodium on urinary excretion of 6-keto-PGF$_{1\alpha}$, iPGF$_{2\alpha}$, sodium and potassium in rabbits (6)

	6-keto-PGF$_{1\alpha}$ (µg day)	iPGF$_{2\alpha}$ (µg/day)	Sodium (mmol/day)	Potassium[b] (mmol/day)
NaCl intake (mean S.E.)	11.8 ± 2.2	2.0 ± 0.6	0.09 ± 0.01	17.6 ± 0.6
h NaCl intake (mean S.E.)	13.3 ± 4.6	0.63 ± 0.10	27.5 ± 3.4	12.8 ± 1.6
ference	NS	p 0.05	p 0.05	p < 0.05

Mean of 24 h urinary excretion from 6 rabbits.
Mean of tw consecutive 24 h excretions from 6 rabbits.

Discussion

The present paper confirms previous reports on the capacity of rabbit renal tissues to form 6-keto-PGF (Pace-Asciak and Rangaraj 1977 Whorton *et al* 1978). Our main finding is the post mortem accumulation of 6-keto-$PGF_{1\alpha}$ in the renal cortex. The level of 6-keto-PGF is here more than 5 times higher than previously reported for PGE_2 and $PGF_{2\alpha}$ (Larsson and Änggård 1976). However in the renal medulla and papilla formation of PGE_2 dominates. Under identical experimental conditions, the renal medulla has been found to synthesize 9 2±2.2 (S D) / g PGE_2/g (Oliw *et al* 1978) *i e* about 5 times more PGE_2 than 6-keto-PGF . Our results therefore indicate that arachidonic acid is metabolized preferentially to primary PGs in the medulla and to 6-keto-$PGF_{1\alpha}$ or prostacyclin (PGI_2) in the cortex.

The level of 6-keto-$PGF_{1\alpha}$ in the rabbit kidney *in vivo* is likely to be much lower. Synthesis of PGs increases rapidly after death (*cf* Frölich *et al* 1976) Data indicates that the in vivo renal levels of primary PGs *in vivo* are in the low ng/g range (Larsson and Änggård 1976).

The physiological function of PGI and 6-keto-$PGF_{1\alpha}$ in the kidney is unknown. The vessels have a high capacity to synthetize PGI (Moncada *et al* 1976). It is tempting to speculate that PGI is formed by the renal vascular endothelium and that it could be important for the renal circulation The effects of indomethacin on renal cortical blood flow and renal distribution were previously attributed to inhibition of the cortical synthesis of primary PGs (Larsson and Änggård 1974 Gerber *et al* 1978 b) Since formation of 6-keto-PGF dominates over primary PGs in the cortex, the renal circulatory effects of indomethacin could also be due to inhibition of PGI synthesis. This is in agreement with the direct effect of exogenous PGI_2, which increases renal blood flow (Gerber *et al* 1978 a).

The cortical synthesis of PGI might be of physiological importance for renin release. Arachidonic acid, the precursor of primary PGs and prostacyclin, increases renin release *in vivo* (Larsson *et al* 1974 Bolger *et al* 1976) and *in vitro* (Weber *et al* 1976), whereas synthesis inhibitors block renin release (Larsson *et al* 1974 Weber *et al* 1976). Interestingly neither PGE nor $PGF_{2\alpha}$ stimulates renin release *in vitro* (Weber *et al* 1976).

The possibility that PGI is the arachidonic product which stimulates renin release has recently gained experimental support *in vivo* (Gerber *et al* 1978 a) and *in vitro* (Whorton *et al* 1977) If this theory is correct one should expect the renal PGI synthesis to change in parallel with the renin-angiotensin system. Sodium deprivation stimulates the renin-angiotensin system and sodium loading has the opposite effect In the present study we found no change in urinary 6-keto-$PGF_{1\alpha}$ excretion when sodium excretion was increased 300 fold, while $PGF_{2\alpha}$ excretion decreased This might indicate that the intrarenal PGI synthesis is not influenced by variations in sodium excretion Another possibility is, however that urinary 6-keto-$PGF_{1\alpha}$ excretion to a large extent reflects the total body 6-keto-$PGF_{1\alpha}$ synthesis rather than the intrarenal synthesis.

The urinary excretion of PGE_2 and $PGF_{2\alpha}$ is generally believed to reflect the intrarenal synthesis. This assumption is based on the very low levels of these PGs in arterial blood, on the high capacity of the kidneys to metabolize PGs coming in the arterial blood and on parallel changes in renal PG synthesis and urinary PGE_2 and $PGF_{2\alpha}$ excretion (Frölich *et al* 1975 Dunn *et al* 1978). It is presently unknown if urinary 6-keto-$PGF_{1\alpha}$ reflects the intrarenal

synthesis. Recent observations indicate that PGI might be a circulating hormone ...cada et al. 1978). Whereas PGE_2 and $PGF_{2\alpha}$ enter the tubular fluid in the loop of ... (Williams et al. 1977), 6-keto-PGF$_{1\alpha}$ might also be filtrated in the glomeruli and it ... enter the tubular fluid at cortical parts of the nephron.

...mmary the present study demonstrates a considerable *relative* 6-keto-PGF$_{1\alpha}$ synthesis ... renal cortex, while PGE_2 and $PGF_{2\alpha}$ are the major PG-endoperoxide products in the ... medulla. Excretion of 6-keto-PGF$_{1\alpha}$ in rabbit urine is 5–10 times higher than urinary ...$_{2\alpha}$ excretion, but unlike $PGF_{2\alpha}$, the 6-keto-PGF$_{1\alpha}$ excretion is not influenced by varia... in sodium intake.

...resent study was supported by grants from the Medical Research Council (2211), Karolinska Insti... ...funder Magnus Bergvalls stiftelse and Svenska Läkaresällskapet. Dr U Axén at the Upjohn ...any generously supplied prostaglandins and deuterated standards.

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Acta physiol. scand. 1979 105, 367–373

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Different effects of furosemide on urinary excretion of prostaglandin E_2 and $F_{2\alpha}$ in rabbits

By

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Abstract

E. and E. Änggård. *Different effects of furosemide on urinary excretion of prostaglandin E_2 and $F_{2\alpha}$ in rabbits.* Acta physiol. scand. 1979 *105* 367–373

...ect of constant infusion of furosemide (130 µg/min i. for 60 min, n = 8) was studied on urinary ...on of water, electrolytes and immunoreactive prostaglandin E_2 (iPGE$_2$) and iPGF$_{2\alpha}$ in chloralose-...anaesthetized rabbits. During the furosemide infusion sodium and water excretion increased ten-...d the excretion of potassium and iPGE$_2$ tw... to three times. The excretion of iPGF$_{2\alpha}$ (0.06 ± 0.03 .../100 g kidney weight) was not significantly changed during the furosemide infusion but increased ...ly after the infusion and reached ... maximum (1.0 ± 0.6 µg/min/100 g) 30 to 45 min later while the ...increase in iPGE$_2$ excretion at this time could be attributed to cross-reaction ...ith PGF$_{2\alpha}$. The results ...st that PGE$_2$ might possibly be involved directly in the action of furosemide, while PGF$_{2\alpha}$ might par... in sodium and ...ater conserving mechanisms in the rabbit kidney activated by the drug induced ...

...ords: Prostaglandin E_2, prostaglandin $F_{2\alpha}$, furosemide, radioimmunoassay, sodium balance

...semide belongs pharmacologically t... the group of diuretics which potent saluretic ...ts are mainly attributed to inhibition of an active chloride transport in the loop of ...e (Seely and Dirks 1977). Furosemide also augments renal blood flow (Williamson 1975) and increases renin release *in vivo* (Vander and Carlson 1969) and *in vitro* ...s and Churchill 1975).

...e present paper will focus on the possible interaction between furosemide and the ...l prostaglandin (PG) system. The renal PGs might be important in regulating renal ...lation, sodium excretion and renin release (*cf.* Dunn and Hood 1977). Many observa-...s indicate that furosemide affects the renal PGs. Williamson *et al.* (1975) demonstrated ...ncrease in renal venous PGE following furosemide in dogs. Furosemide also increases ...ary prostaglandin excretion in normal man and in hypertensive patients (Abe *et al.* ... Weber *et al.* 1977 a, Scherer *et al.* 1978). Evidence for a connection between the ...n of furosemide and the renal PGs has been obtained by the use of PG synthesis ...bitors.

Acetylsalicylic acid and indomethacin were found to block the effect of furosemide on renal blood flow and renal venous PG release in dogs (Williamson et al. 1975, Bey and Loew 1977). Indomethacin also reduced the effect of furosemide on plasma renin activity and on sodium excretion in man (Patak et al 1975, Rumpf et al 1975, Frolich et al 1976) and in anesthetized animals (Oliw et al 1976), although the inhibition of sodium excretion is less pronounced in hydrated animals (Kövér and Tost 1977, cf Bailie et al 1976).

These observations indicate that the effects of furosemide could be partly mediated by the renal PGs. The present report was designed to study the action of furosemide on urinary PG excretion. Rather unexpectedly we found a temporal difference in the effect of furosemide on urinary PG excretion. The increase in PGE excretion was associated with the diuresis whereas the increase in $PGF_{2\alpha}$ excretion appeared when the drug effect was over. These results indicate that PGE and PGF compounds could have different roles in the kidney.

Material and method

Materials

All reagents were of analytical grade. Furosemide (Hoechst, Frankfurt, West Germany) was dissolved in 0.05 M sodium phosphate buffer pH 8.0 (1.0 mg/ml) and infused at a constant rate of 8 ml/h. Indomethacin (Merck, Sharp and Dohme, Rahway, New Jersey) was dissolved in ethanol (0.5 ml). ^{3}H PGF (150 Ci/mmol) and ^{3}H PGE_2 (150 Ci/mmol) for use in radioimmunoassay were obtained from New England Nuclear, Darmstadt, West Germany. Antibodies against $PGF_{2\alpha}$ or $PGF_{2\alpha}$-albumin complexes were raised in rabbits and characterized as previously described (Oliw et al. 1978 a). The Ringer solution contained (mM): 150 NaCl, 2.3 $CaCl_2$ and 3.5 KCl.

Experimental

Male white New Zealand rabbits (3.0±0.6 (S.D.) kg) were kept on a standard pellet diet (R 11, Astra-Ewos, Södertälje, Sweden) and water *ad libitum*. The rabbits were anesthetized by infusion in a marginal ear vein of a luke-warm Ringer solution (10 ml/kg) containing chloralose (8 mg/ml) and urethane (50 mg/ml). The rabbits were then left undisturbed for 30 min. Sometimes an additional amount (2-4 ml) of anesthesia was given before a tracheal cannula was inserted. Blood pressure was monitored from the right carotid artery by means of a Statham pressure transducer (P23DC) and a Grass polygraph. The left external jugular vein was used for infusion of Ringer solution at a constant rate of 0.8 ml/min throughout the experiment.

A low abdominal incision was made and the bladder was emptied. Both ureters were cannulated 1-2 cm with polyethylene tubing (Intradermic PE 50), the tip of which had been carefully blunted by scrubbing against paper. In spite of these precautions, hematuria sometimes occurred. Urine was collected in chilled glass tubes containing 50 µg indomethacin to prevent *in vitro* PG synthesis from formed elements of blood. The urine samples were centrifuged at +4°C (15 min) at the end of each collection period and frozen at the end of the experiment.

The experiment was started 60 min after beginning of the Ringer infusion. After two control periods (15 min, 2), furosemide (n=8) or solvent (n=3) was infused into a marginal ear vein during 4 periods (15 min, 4) and urine was then collected for 2 periods in 3 expts. and for 6 periods in 5 expts. During this time the animals were neither artificially ventilated nor heparinized but a few ml of chloralose-urethane was sometimes needed. At the end of the experiment the kidneys were freed from surrounding tissues and weighed (mean weight 10.3±4.5 (S.D.) g).

Analytical methods

Sodium and potassium in urine were measured by flame photometry using lithium as an internal standard. PGE_2 and $PGF_{2\alpha}$-like immunoreactivity were measured in unextracted urine as previously described (Oliw et al. 1978 a). $PGF_{2\alpha}$ was measured by radioimmunoassay (RIA) using $PGF_{2\alpha}$-antiserum and the values obtained are referred to as immunoreactive $PGF_{2\alpha}$ ($PGF_{2\alpha}$). The assay has been validated for rabbit urine by direct comparison with GC-MS analysis with good agreement between the two methods (Oliw

978) For the PGE_2 analysis, the urine samples were treated with NaBH converting PGE_2 to PGF $GF_{2\alpha}$. The latter is not naturally occurring in the rabbit and could therefore be used to calculate of $PGF_{2\alpha}$ was measured by $PGF_{2\alpha}$-antiserum and the values for immunoreactive $PGF_{2\alpha}$ are used as a of PGE_2.

sign test was used for statistical analysis (Dixon and Massey 1957) and data given as mean ± S.E. the urinary excretion are expressed per 100 g kidney weight.

Results

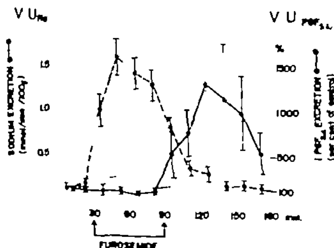
on sodium, potassium and water excretion

semide increased urine flow and sodium excretion from 1.0 ± 0.3 and 0.16 ± 0.04 respectively to a maximum of 13.0 ± 1.2 ml/min/100 g and 1.6 ± 0.2 mmol/min/100 g, respectively between 15 and 30 min of infusion (Fig. 1). Already 15 to 30 min after ending furosemide infusion, the urine flow had decreased to 2.6 ± 0.6 ml/min/100 g and sodium excretion to 0.37 ± 0.08 mmol/min/100 g.

The urine losses were not replaced but the animals were infused with 0.8 ml/min of ter solution. The sodium balance (sodium input minus the sodium output during the semide diuresis, *i.e.* from 30 to 105 min) was negative in 7 of 8 expts. and averaged 58 0.13 mmol/min/100 g.

Potassium excretion was increased 3 times by furosemide from 0.03 ± 0.01 to a maximum of 0.10 0.15 mmol/min/100 g and decreased to control levels after the infusion (Fig. 2).

the controls receiving solvent of furosemide, mean urinary water and sodium excretion ranged between 2.0 and 5.4 ml/min/100 g and 0.31 and 0.45 mmol/min/100 g. Mean urinary potassium excretion ranged between 0.04 and 0.07 mmol/min/100 g.



1. The effect of furosemide infusion (130 µg/min for 1 h) on the urinary excretion of sodium and immunoreactive $PGF_{2\alpha}$ ($iPGF_{2\alpha}$). The vertical lines mark the beginning and the end of the infusion. Sodium excretion (○—○). Excretion of $PGF_{2\alpha}$ (●—●) in per cent of the excretion in the control periods before infusion. Mean ± S.E. (n = 5–8). The mean excretion of PGE_2 in per cent of control is indicated by the dotted line.

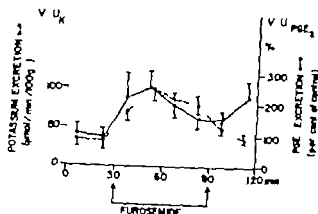


Fig. 2. The effect of furosemide [illegible] the excretion of potassium and [illegible] the PGE (PGE). PGE is reduced [illegible] N BH treatment to $PGF_{2\alpha}$ and PGE [illegible] the latter measured by [illegible] Potassium excretion (O—O) and PGE excretion in per cent of control (●--●). The increase in PGE_1 excretion after the [illegible] semide infusion could be attributed to cross reaction between the $PGF_{2\alpha}$-antiserum [illegible] PGF (cf Fig. 1 and the text).

Effect on urinary PG excretion

Urinary excretion of $iPGF_2$ was not significantly changed during the furosemide infusion. The $iPGF_{2\alpha}$ excretion was 0.06 ± 0.03 μg/min/100 g before infusion, 0.03 ± 0.01 ($p > 0.05$) after 15 to 30 min of infusion and 0.03 ± 0.01 ($p > 0.05$) at the end of the infusion. It then increased in 7 of 8 expts. and was 0.34 ± 0.19 μg/min/100 g 15 to 30 min later ($p < 0.05$) and reached a maximum of 1.0 ± 0.6 μg/min/100 g ($n = 5$) in the next urine collection period. The relative changes in iPGF excretion are demonstrated in Fig. 1. There was no correlation between sodium losses during furosemide diuresis and the subsequent $iPGF_{2\alpha}$ excretion but iPGF excretion did not increase in the experiment with positive sodium balance during the furosemide diuresis.

Furosemide increased urinary iPGE and potassium excretion in parallel (Fig. 2). The iPGE excretion increased from 4.6 ± 1.3 to a maximum of 11.7 ± 2.7 ng $iPGF_{2\alpha}$/min/100 g after 15 to 30 min of infusion ($p < 0.05$) and was 7.1 ± 1.3 ng/min/100 g at the end of the infusion. The relative changes in iPGE excretion in per cent of control is indicated in Fig. 2 (dotted line). During the marked $iPGF_{2\alpha}$ excretion the urinary output of $iPGE_1$ did not exceed 12 ng/min/100 g. However the antiserum which was used for $PGF_{2\alpha}$ analysis cross reacts to about 1% with $PGF_{2\alpha}$ (Oliw *et al* 1978 a). The increase in $iPGE_1$ could therefore be totally attributed to cross reaction with $PGF_{2\alpha}$ in the samples.

In controls ($n = 3$) no significant changes were noticed in $iPGE_1$ and $iPGF_{2\alpha}$ excretion which is an agreement with previous studies (Oliw *et al* 1978 a).

Mean blood pressure

Mean blood pressure fell slightly during the experiment from 107 ± 5 mmHg before, to 93 ± 4 at the end of the furosemide infusion ($p < 0.05$) and was 92 ± 4 mmHg ($n = 8$) and 96 ± 6 mmHg ($n = 5$) 30 and 75 min later. Mean blood pressure also decreased slightly in the 3 control expts.

Discussion

PGE_2 and $PGF_{2\alpha}$ in urine originates from the kidneys and urinary excretion of these PGs can be used to estimate the intrarenal PG synthesis (Frolich *et al* 1975, Williams *et al* 1977, Dunn *et al* 1978). The present study demonstrates that furosemide increases urinary PGE_2 excre-

2 3 times, thus considerably less than the concommitant increase in sodium and water
... tion. This is in agreement with recent studies by Abe *et al.* (1977) and Scherer *et al*
...), who found a similar increase in PGE_2 excretion by furosemide in normal and in
...rtensive humans. Weber *et al* (1977 a) and Scherer (1978) also found a large increase
... $F_{2\alpha}$ excretion in normal humans. The main finding in our study is that the increase
...rinary $PGF_{2\alpha}$ excretion follows *after* the furosemide diuresis. Because of long urine
...ction intervals in previous studies, the late increase in $PGF_{2\alpha}$ excretion might have been
... looked.

...he mechanism by which furosemide increases the urinary PG excretion is likely to be
...plex. One explanation has been an inhibition of PG metabolism in the kidney. Thus
...semide has been proposed t reduce PG inactivation by inhibiting the enzyme 15-
...roxy-PG dehydrogenase (Paulsrud and Miller 1974 Abe *et al* 1976). However ID_{50}
... the inhibition of swine kidney 15-hydroxy-PG-dehydrogenase by furosemide is in the
...ge of 10^{-4} M (Oliw unpublished). There are also no evidence that furosemide directly
...bits PG metabolism *in vivo* (Gerber *et al* 1978).

...urosemide has also been proposed to stimulate PG synthesis. Weber *et al* (1977 a)
...nd that furosemide increased plasma arachidonate i man. They reasoned that the plasma
...chidonate in turn increased the renal PG synthesis. However this cannot explain the
... increase in $PGF_{2\alpha}$ in our experiments. In view of the short duration of action of furo-
...ide the late increase in $PGF_{2\alpha}$ excretion in our study is more likely to be secondary to
...ium and water losses rather than to a direct effect of furosemide. This explanation would
... be more consistent with the increased $PGF_{2\alpha}$ excretion in the sodium deprived animal
... below).

...revious studies have shown that dietary sodium intake influences renal PG synthesis.
...iet low in sodium chloride increases the intrarenal PGE_2 levels in rats and a diet high in
...ium chloride decreases them (Tobian and O'Donnell 1976). Similar changes also occur
...urinary PG excretion in rabbits (Weber *et al.* 1977 b Davila *et al.* 1978). Furthermore,
...racellular fluid olume expansion with Ringer solution decreases urinary $PGF_{2\alpha}$ excre
... in rabbits (Oliw *et al.* 1978 b). These observations indicate that the late increase in
...$F_{2\alpha}$ excretion following furosemide might be related to the negative sodium balance.
... natriuretic action of the renal PGs has recently been proposed (Tobian and O'Don-
...ll 1976, Kirschenbaum and Stein 1976, 1977).

Another possibility is that furosemide could also increase PG synthesis by stimulating
...e renin-angiotensin system. Angiotensin II has been found to increase urinary PG excre
...n *in vivo* (Frölich *et al* 1975, Dunn *et al* 1978) and PG synthesis *in vitro* (Danon *et al*
...75). The renin-angiotensin system, as well as anesthesia and other vasoconstrictor stimuli,
...ght therefore contribute to the increased urinary PG excretion in our study (Scherer *et al*
...77).

In the present study furosemide increased potassium and PGE_2 excretion in parallel
...owever this might not be a causal relationship, since urinary PGE_2 and $PGF_{2\alpha}$ excretion
...re not changed by potassium deprivation in rats (Hood and Dunn 1978).

In summary our data suggest that the early increase in PGE_2 excretion could be related
... the effects of furosemide on renal blood flow (Williamson *et al* 1975 Berg and Loew

1977) The late and large increase in urinary $PGF_{2\alpha}$ excretion could be attributed to renal compensation of sodium and water losses. The major mechanism of furosemide is to dissipate the electrolyte concentration gradient in the medulla by blocking an active chloride transport (Olsen 1975) Indomethacin on the other hand increases this gradient (Ganguli et al. 1977). It is therefore tempting to speculate that $PGF_{2\alpha}$ is somehow related to the maintenance of the electrolyte gradient in the renal medulla.

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Fasting and prolonged exercise increase vasoactive intestinal polypeptide (VIP) in plasma

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Abstract

GALBO, H., J HILSTED J FAHRENKRUG and O B. SCHAFFALITZKY DE MUCKADELL *Fasting and prolonged exercise increase vasoactive intestinal polypeptide (VIP) in plasma* Acta physiol. scand 1979 *105* 374–377

6 young men had venous blood drawn during 4 experiments. The concentration of VIP in plasma increased markedly (from 1 8 (0–4.5) to ...3 (7 8–43 8) pmol l^{-1} mean and range) during 3 h of mild bicycle exercise but not at all during an equivalent period of rest or during short term submaximal and maximal exercise. During 59 h of fasting, VIP increased from 3 6 (0.6–6.6) to 10 2 (6 6–1 ...) pmol l^{-1} ($p < 0.05$). The concentration of glucose in plasma decreased significantly during the prolonged exercise as well as during fasting. The known metabolic actions of VIP and the demonstrated increase in its plasma concentration during negative energy balance indicate that VIP is a polypeptide of substrate ...

Vasoactive intestinal polypeptide (VIP) was originally isolated from porcine small intestine (Said and Mutt 1970) It is considered as a possible gastrointestinal hormone (Rayford, Miller and Thompson 1976 a, b Johnson 1977) and since recently as a possible neurotransmitter (Bryant *et al* 1976, Larsson *et al* 1976) Because of similarities in amino acid sequence and pharmacology VIP has been grouped together with glucagon, secretin and gastric inhibitory polypeptide into the secretin-glucagon family (Rayford, Miller and Thompson 1976 a, b, Johnson 1977) However the physiological role of VIP is not established (Rayford, Miller and Thompson 1976 a b, Bryant *et al* 1976, Johnson 1977) In order to clarify the physiological role of a hormone it is necessary to elucidate the stimuli for its release The concentration of glucagon in plasma has been shown to increase during muscular exercise (Galbo, Holst and Christensen 1975) and fasting (Marliss *et al* 1970) In order to extend the knowledge about the physiology of VIP we have now measured its concentration in plasma during these strains.

Materials and methods

6 healthy males (25 (17 31) yrs (mean and range), 183 (176–187) cm and 77 (6...–81) kg), familiar with the laboratory and the applied procedures, volunteered in the study At intervals of 1 7 weeks they participated in 4 different experiments the sequence of which was ...ned One morning the subjects exercised 3 h at ...

-ally braked bicycle ergometer at a load (70–147 W) calculated to require 40% of the individual al oxygen uptake (V_{O_2} max, 56 (52 59) ml kg⁻¹ min⁻¹) determined previously on treadmill. —r morning the subjects rested in chair for an identical period of time. Still another morning the -s performed tw successive treadmill runs (inclination 7°) lasting 10 and 5 min and previously ned to require 60 (speed 6 4–7 5 km h⁻¹) and 100 % (9.5–11 7 km h⁻¹), respectively of individual m. Finally the subjects were examined after 59 h fast during hich they were not allowed to smoke -rpate in sports and only consumed water mineral water, vitamins and tea. The subjects always the laboratory after good night's sleep, at least 10 h fast (only taking in water) and tobacco -ee and after at least 32 h alcohol and sports abstinence. They were weighed and had catheter -d in forearm vein, hereupon they rested 30 min before the first blood sample was drawn. (I - exercise expts the catheter was advanced to the subclavian vein). The electrocardiogram was -ed ch precordial electrodes. Blood for VIP determinations was collected in iced tubes containing of heparin and 500 KIU of aprotinin per ml of blood. After centrifugation at 4°C plasma was stored) C until analysis by specific, sensitive and precise radioimmunoassay (Fahrenkrug and Schaffalitzky -dadell 1977). Glucose (Schmidt 1961) and glycerol (Eggstein 1966) were determined by enzymatic -photometric methods and FFA spectrophotometrically as copper soaps (Duncombe 1964). -l cell volume was determined by the microhematocrit method. Statistical evaluation of the data -ade by means of Wilcoxon nonparametric ranking test for paired data and Spearman rank -tion analysis (Siegel 1956).

Results

-ng the prolonged exercise the concentration of VIP in peripheral plasma increased -ssively (Fig. 1). Simultaneously plasma glucose decreased (Fig. 1) and was at the end of -se inversely correlated with the markedly increased VIP concentrations (r_s = −0.886, -.05). Heart rate increased throughout the exercise and was 135 (133–138) and 145 (14?– -) beats min⁻¹ (p 0 05) in the 30'th and in the 180'th min, respectively The hematocrit -ased slightly from 43.9 (40 7–47.3)% at rest to 45.5 (42.5–48 1)% at the end of -se (p 0 05). During the 3 h of rest in the control expt. the measured parameters did - change significantly

-ring short term moderate and heavy exercise neither VIP nor glucose concentrations -were changed significantly VIP concentrations were 3 6±0.9 3 7±1 1 and 4 4±1 1 - l⁻¹ mean and S E at rest, submaximal and maximal exercise, respectively while -se concentrations ere 5.2±0.1 5.1±0.2 and 5.2±0.2 mmol l Heart rate increased - 76 4 at rest to 152 6 and 189±2 beats min during submaximal and maximal -cise, respectively while the hematocrit was 43 4±0.8 45.3±0.8 and 47.0±0 8 -(0 05)% respectively

-fter 59 h of fasting the subjects had lost 3 (–4) kg of body weight and had pronounced -nuria measured by stix (Ames). The concentrations in serum of FFA (from 0.302 -(0.45-0 491) to 0.980 (0 622 1 742) meq l) and glycerol (from 5? (39–67) to 178 (122–281) -l) had increased (p 0 05) Also VIP concentrations in plasma had increased -ficantly hile glucose concentrations had decreased (Fig. 2). Hematocrit was 43 4±0.8% -re and 45 7 1.3 after the fast (p 0 1).

Discussion

present study has shown that the concentration of immunoreactiv VIP in peripheral -ma increases during fasting and during prolonged exercise of an intensity equivalent - everyday activities. During short term moderate or heavy exercise, however VIP

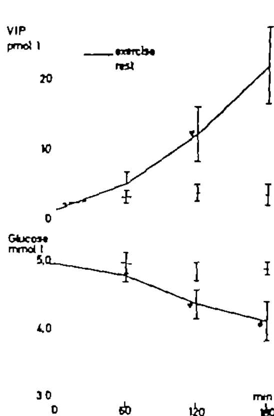


Fig. 1

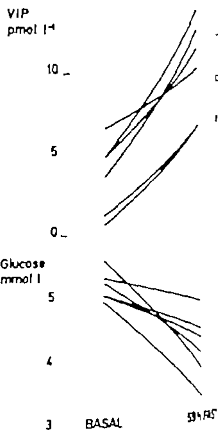


Fig. 2

Fig. 1 Mean plasma concentrations (± S.E.) of vasoactive intestinal polypeptide (VIP) and of glucose ... 6 subjects during rest and prolonged exercise are plotted against time. denotes value significantly different from initial value ($p < 0.05$). ∇ denotes value significantly different from value obtained after an equal period of rest ($p < 0.05$)

Fig. 2. The plasma concentrations of vasoactive intestinal polypeptide (VIP) and of glucose ... standing subjects after an overnight fast (Basal) and after 59 h of fasting.

concentrations did not change significantly Similar findings have been made in the case of glucagon (Marliss *et al* 1970 Galbo, Holst and Christensen 1975). Since VIP is widely distributed in the body (Bryant *et al* 1976 Larsson *et al* 1976) the origin of the VIP molecules causing the increased concentrations in plasma can not be pointed out with certainty. However the highest tissue concentrations of VIP have been found in the gastrointestinal tract (Bryant *et al* 1976, Larsson *et al* 1976) VIP may be released from VIP-containing fine nerve fibers (Bryant *et al* 1976, Larsson *et al* 1976) and endocrine cells (Bryant *et al* 1976) The fact that VIP-immunoreactive endocrine cells have not been detected in ... by the VIP antiserum used to measure VIP concentrations in the present study (Larsson *et al* 1976) indicates that VIP in plasma derived from nerve fibers.

The consequences of an increased release of VIP during exercise and fasting can not be pointed out with certainty either The pharmacology of VIP comprises many actions (Rayford, Miller and Thompson 1976 a, b Johnson 1977) Among these stimulation of glucagon secretion (Ohneda *et al.* 1977), lipolysis (Frandsen and Moody 1973), and hep...

genolysis and gluconeogenesis (Matsumura, Akiyoshi and Fujii 1977) appear most ant in relation to the observed increases in VIP concentrations during exercise and ng. Certainly VIP is hardly produced in the liver (Bryant *et al.* 1976) but since VIP is ly inactivated on hepatic transit (Rayford, Miller and Thompson 1976 a), the entration of VIP in portal blood draining the gastrointestinal tract possibly in the nt study was higher than in peripheral blood and sufficient to stimulate hepatic ogenolysis and gluconeogenesis. Higher concentrations of VIP in portal than in peri-al blood have previously been found in anesthetized pigs (Fahrenkrug *et al.* 1978). The lected biological actions and the finding in the present study of increased plasma entrations during negative energy balance indicate that VIP like glucagon, has a iological role as "a polypeptide of substrate need".

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Effect of terbutaline on lecithin content in alveolar lung wash in fetal rabbits

By

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Adrenergic beta-receptor stimulating drugs have been used extensively during the last years in the treatment of threatening preterm labour There has also been a considerable interest in the effects of these drugs on the fetus and neonate, especially on the lung function of the premature infant Clinical studies indicate a reduced incidence of respiratory distress syndrome in premature infants born of mothers who have been treated with beta-mimetics such as isoxuprine or ritodrine (Kero *et al* 1973 Boog *et al* 1975). Bergman and Hedner (1978) used a more selective $beta_2$-receptor stimulating drug, terbutaline, in an attempt to arrest preterm labour and found a reduced incidence of hyalme membrane disease in a series of preterm infants

Wyszogrodski and associates (1974) demonstrated improved pulmonary stability by pressure-volume experiments on lungs of isoxuprine injected fetal rabbits. We have found an increased pulmonary distensibility in similar experiments after terbutaline administration into fetal rabbits 3 h before preterm delivery (Bergman *et al* 1978) These results indicate an increased pulmonary maturation after administration of beta mimetics. However a more direct way to assess the effect of these drugs on pulmonary maturation is to measure certain biochemical parameters of surfactant in the fetal lung. Enhörning *et al* (1977) investigated the surfactant properties after isoxuprine administration to fetal rabbits and found an increased lecithin/sphingomyelin ratio of the fetal pulmonary fluid.

The purpose of the present study was to investigate the effect of terbutaline on the lecithin content in alveolar wash from the preterm rabbit lung.

New Zealand white rabbits, weighing betwee 4 1 and 4.5 kg, were mated under direct observation [illegible] 28 days f gestation half of the does received an tramuscular njection of 2.5 mg terbutaline per kg [illegible] The control animals received an equal volume of vehicle. 3 h after the injection the does were anaesthetized with ther and a laparotomy was perf rmed The f tuses were removed, immersed normal saline and [illegible] allowed t breathe during this procedure After tracheotomy a cannula was inserted and the lungs [illegible] expanded with 2 ml air This was followed by 6 gentl lavagings, each with 1 ml of 0.9 saline [illegible] The cumulative lecithin recovery by this method is pproximately 95% (Gluck *et al* 1967). Lung [illegible] lecithin was extracted with chloroform, metha l (1 2). The chloroform layer was chromatographed [illegible] thin-layer silica gel plates. The lecithin spot were charred by sprayi g with copper acetate reagent and [illegible] concentration of lecithin determined by densitometric scan g (Zeiss p4) (Fewster *et al* 1969).

I. Details of the material.

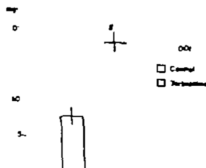
	Control	Terbutaline
ber of does	3	3
ber of fetuses	16	23
l weight		
an ± S.E.)	42.2±0.8	43.2±1.3

o significant difference in fetal weight was found between the two groups. The mean l weight was 43.2 g in fetuses of terbutaline treated mothers compared to 42.2 g in the trols (Table I).

he total lecithin of pulmonary lavage of fetuses born of terbutaline-treated does was ficantly higher (p 0.001) than the total lecithin in the lavage fluid from fetuses of saline ated does (Fig. 1).

he lung ash lecithin content is known to reflect the pulmonary biochemical maturan the fetal rabbit. The lecithin level in pulmonary washings increased by approximately after terbutaline administration to the does, which is in agreement with the corresnding results of Enhörning *et al.* (1977) who administered isoxuprine directly into the uses. It has also been shown that beta-receptor stimulating drugs, such as terbutaline, n to the pregnant rabbit, reaches the fetuses in small amounts (Bergman *et al.* unlished observations)

he exact mechanism whereby terbutaline affects the maturation of the fetal lung is not wn at present. The data achieved may be the result of a variety of mechanisms. Beta rgic drugs are known to effect a dilatation of the lower airways and recently also a hydration of the fetal lungs has been demonstrated (Enhörning *et al.* 1977). These two tors may facilitate neonatal respiratory adaptation. However the increased amount of al lecithin recovered from pulmonary lavage could hardly be explained by dilatation and hydration. It seems more logical to assume that the increased lecithin levels reflect the chanism earlier proposed, namely that of a release of intracellularly stored surfactant o the alveolar space. This explanation seems attractive in view of the fact that the fetal g at this stage of gestation is morphologically mature, but that surfactant is still mainly



g. 1. Effect of maternal terbutaline on total lecithin ment in alveolar lung wash in fetal rabbits. Show are ± S.E. Figures indicate the number of experiments.

stored intracellularly (Wyszogrodski *et al* 1974 Kikkawa *et al.* 1968). The phenomena described can also be explained by an increased biosynthesis of pulmonary lecithin as beta-receptor stimulating agents are known to increase tissue cyclic adenosine monophosphate, which could lead to enhanced pulmonary lecithin synthesis (Barrett *et al* 1976)

If the physiological situation and the effect of terbutaline are similar in the human fetus there seems to be reason to consider this beneficial influence of beta-mimetics by the treatment of preterm labour and also when evaluating the effect of corticoid treatment on lung maturation The possible clinical value of terbutaline in this respect is supported by the results of a recent clinical report (Bergman and Hedner 1978). However before definite conclusions are made it should also be born in mind that any beneficial effects of beta-mimetics depend on the placental passage as related to dose.

This study was supported by grants from the Swedish Medical Research Council (no 2464), The Medical Faculty University of Göteborg and Göteborgs Läkaresällskap The technical assistance by Mrs Lena Nystrom, Britt Marie Otterstedt and Gabriella Salén is gratefully acknowledged

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Effects of angiotensin II on cerebral blood vessels

By

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octapeptide angiotensin II exerts a potent constrictor action on vascular and non-vascular oth muscle (Page and Bumpus 1974 Page and McCubbin 1968) by a direct action on muscle cell and by a potentiating effect of adrenergic nerve activity (Zimmermann 1978). iotensin receptors have been demonstrated on the plasma membrane of many target es g aorta (Altura and Altura 1977 Baudouin t al. 1972, Devynck et al 1973 LeMor and Palaic 1975). Besides the direct vasoconstrictor action of angiotensin other central ts of the peptide following intraventricular infusions have been demonstrated (see fman et al 1977) an antidiuretic hormone release, drinking and a centrally mediated sor response. This is in accordance with recent demonstrations of the existence of iotensin II-containing nerve terminals in brain (Fuxe et al. 1976) This would indicate a sad ty of action of angiotensin II on brain vessels whereby central release of the peptide ht induce a local vasoconstriction. The peptide has for long time been used in studies the cerebral blood flow particularly in the phenomenon of autoregulation, which is the inate ability of the brain to maintain a constant flow despite relatively marked changes he perfusion pressure (see Edvinsson and MacKenzie 1976) In such studies it is assumed t angiotensin II, used to induce the desired degree of hypertension, is in itself inert with pect to the cerebral circulation. Evidence that this is not true has been obtained in the sent study of a model utilizing isolated middle cerebral arteries from cats on which the scular action induced by angiotensin II has been tested and the response compared with ban other asoactive agents.

Isolated segments (5 mm long, 300–400 μm in diameter) of cat s middle cerebral artery re obtained from 6 adult cats of either sex (3 cats were subjected to cranial sympathectomy 1 week beforehand). The animals were exsanguinated under nembutal anesthesia (30 mg/kg i.p.), the brain was removed, and the vessels immediately dissected out and placed in aerated Krebs-Ringer buffer solution of the following composition (mM concentrations) NaCl 118 KCl 4.5 $MgSO_4$ $2H_2O$ 1.0 KH_2PO_4 1.0 $NaHCO_3$ 25 $CaCl_2$ H_2O glucose 6.0 Two preparations were mounted between metal prongs in the same mantled gan bath, continuously aerated with 95 % O_2 and 5 % CO_2 to give a pH between 7.3 and 4 Circular mechanical activity was measured by Endevco Model 8107 2 force-displacement transducers and recorded on a Grass Model 7B polygraph. The technique has previously en described in detail (Ed insson t al 1974 Edvinsson and Owman 1974).

Administration of angiotensin II amide (Hypertensin, CIBA) into the organ bath induced

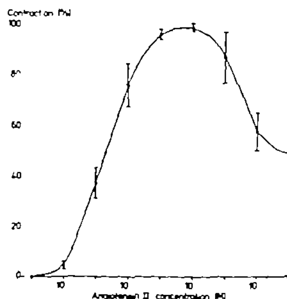


Fig. 1

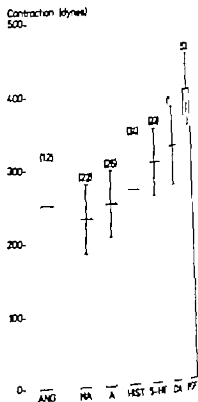


Fig. 2

Fig. 1 Log dose-response relationship following cumulative applications of angiotensin II to cerebral arteries in 12 expts. The values show mean contractions (expressed as percent of the maximum response) ± S.E.

Fig. 2. Comparison between maximum contractile effects of angiotensin II (ANG) and that of noradrenaline (NA), adrenaline (A), histamine (HIST), 5-hydroxytryptamine (5-HT), dopamine (DA) and prostaglandin $F_{2\alpha}$ (PG$F_{2\alpha}$) compiled from data obtained in previous studies (Edvinsson and Owman 1977). Values are means ± S.E., number of expts. within parenthesis.

a prompt constriction of the isolated segments of the middle cerebral artery. The response was the same irrespective of whether the vessels had been sympathectomized or not before hand. Therefore all dose-response curves were grouped together. A constriction was produced already at a concentration of 10^{-9} M of angiotensin and the maximum effect was obtained with 10^{-} M. In concentrations above 10^{-} M the contractile effect was less marked (Fig. 1). The mean concentration of angiotensin at which half maximum response was obtained was $(5.66 \pm 1.44) \cdot 10^{-9}$ M (mean values ± S.E.) and the maximum contractile effect was (25... ± 61) dynes. Tachyphylaxis often occurred when repeated dose-response curves were made at short time intervals. The contraction did probably not involve an interaction with postsynaptic α receptors since in 2 vessels the angiotensin-induced contraction was the same as in controls after pretreatment during 30 min with 10^{-6} M phenoxybenzamine.

Angiotensin II is of interest not only by itself but also in comparison with other vasoactive hormones. This peptide was slightly less effective as a contractile agent (see Fig. 2) than 5-hydroxytryptamine, dopamine and prostaglandin $F_{2\alpha}$. Angiotensin II is commonly

lerating the systemic blood pressure when studying not only the pressure flow relation- in the cerebral circulation, but also the impaired structural blood–brain barrier func that may follow an acute rise in systemic pressure. In such experiments it is assumed angiotensin has little or no action on the cerebral arteries. However a pronounced raction was obtained with angiotensin II in isolated brain vessels, the effect being ac panied by a gradual acceleration of spike activity (Lusamvuku *et al* 1979). Recently otensin was demonstrated to be a potent constrictor of pial precapillary vessels (Wei 1978). These findings suggest a role of angiotensin II on cerebral essels *in situ* and a ible modulatory involvement in studies on cerebral blood flow autoregulation, although ermeability across the blood–brain barrier is poor (Volicer and Loew 1971)

work as supported by the Swedish Medical Research Council (Grant No. 04X 732).

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Potential clamp experiments on myelinated nerve fibres from alloxan diabetic rats

By

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Diabetic neuropathy in man belongs to the group of neuropathies characterized by segmental demyelination (Thomas and Lascelles 1966) The mechanism for the development of this pathological change is not known Investigations of rats with experimentally induced diabetes have shown that the animals get a reduced conduction velocity (Eliasson 1964, Sharma and Thomas 1974) but the decreased conduction velocity was not related to structural changes as found in the human neuropathy (Sharma and Thomas 1974). The present report describes changes in the nodal function that were observed in potential clamp experiments on single myelinated nerve fibres isolated from the alloxan diabetic rat.

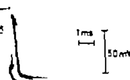
Diabetes was induced by intraperitoneal injection of a buffered solution of alloxan monohydrate (55–150 mg/kg b.w.t.). The animals were maintained on commercial standardized pelleted rat diet and water *ad libitum*. Large single myelinated nerve fibres (10–15 μm) were isolated from the sciatic nerve of diabetic rats (Sprague-Dawley 250 g). Nerves were taken at the onset of weight loss and development of cataract, 7 months after the induction of diabetes. The method for potential clamp of single myelinated nerve fibres was similar to that developed by Dodge and Frankenhaeuser (1958) and has been described in an investigation of normal rat myelinated nerve fibres (Brismar 1978). The experiments were performed at about 25 C and the isolated nerve was kept in oxygenated Ringer solution (147 mM NaCl, 5.9 mM KCl, 3.1 mM CaCl and 5.0 mM Trizma pH 7.4 at 25 C). The isolated single fibre was mounted in a recording chamber and connected to the feedback amplifier system which was used firstly to record the membrane action potential and then to record the membrane currents associated with step changes in the membrane potential.

Fig. 1 shows records of the action potential in a fibre from a diabetic rat. This action potential was followed by a prolonged afterdepolarisation not observed in normal fibres.

The nodal membrane potential was clamped and recordings were made of the membrane current associated with step changes in membrane potential. Plots of the initial current (I_{Na}) vs membrane potential (U) curve in diabetic rat fibres indicated a similar dependence on potential of the Na permeability mechanism as in the normal fibre.

Large differences were found in the K permeability properties between diabetic and normal rat fibres. The delayed outward current during positive potential steps was large in the diabetic rat nerve (Fig. 2) and small in normal fibres (Brismar 1978). Also in the diabetic rat fibre the repolarisation after the positive pulse was associated with a large inward current tail which decreased with a slow exponential time course. These findings were consistent with a large delayed potential dependent increase in the K permeability and a high extranodal [K]. Fig. 3 shows the effect of pulse duration on the amplitude of the delayed outward current and on the current tail at repolarisation. Several current traces

Key words: Potential clamp, myelinated nerve, diabetes, neuropathy



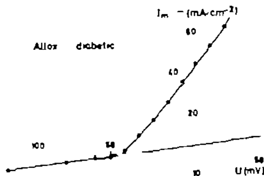


Fig. 1 Fig. 2

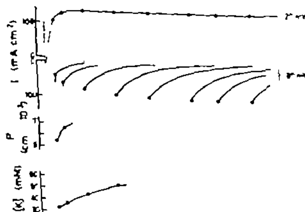
Membrane action potentials and subthreshold responses in alloxan diabetic rat nerve fibre. Stimulus ... were) repeated ... frequency of 1 Hz. Temp 26 C.

... Steady state current vs potential relation in alloxan diabetic rat nerve fibre. Smooth curve fitted ... symbols. Straight line indicates leak current. Temp 26°C.

... used ... th pulses of various duration have been superimposed. U was 20 mV during ... pulse and at the resting level (−80 mV) before and after. The delayed outward current ... used rapidly after the initial Na current and decreased slightly thereafter whereas the ... ent tail associated with repolarisation gradually increased in size with lengthening ... the pulse.

... hese results were analysed on the assumptions that (*i*) the delayed currents and the ... ent tail were carried by K (*ii*) that the instantaneous K permeability (P_K) after a step ... ge in U was equal to the P immediately before the step, (*iii*) that the relation between ... and P satisfied the constant field equation (Hodgkin and Katz 1949 Dodge and ... nkenheimer 1959) and (*iv*) that the $[K]_i$ was 153 mM. On the basis of these assumptions, ... and $[K]_e$ (extranodal [K]) were calculated from the measured outward I_K and inward ... ail associated with various pulse durations. Corresponding values of I_K, P_K and $[K]_e'$ at ... erent times are indicated by symbols in Fig. 3. The calculated change in P ... er pulse

... 3 *Top*. Outward I_K and ... and I_K-tails in alloxan diabetic ... nerve fibre demonstrated by ... superimposed records of membrane currents associated ... with pulses of various duration ... Temp 26 C. Symbols refer to ... times of measurement for the ... calculation of P and $[K]_e'$ (extranodal [K]). *Middle* and *bottom* ... and $[K]_e'$ calculated from I as ... described in text. Smooth curves ... fitted to symbols.



duration showed an S-shaped increase towards a ceiling level (P_K 7.0 cm s⁻¹ 10⁻⁶) which was maintained during the pulse This value is considerably higher than found in normal rats (0.2 cm s⁻¹ 10⁻⁶ Brismar 1978) The calculations further indicated a large increase in [K]ₒ during the positive potential steps. This finding may explain the high value of I_K in the I_K vs U curve in Fig. 2 and the presence of an afterdepolarisation (Fig. 1).

A large increase in the amplitude of the delayed current was found in 3 alloxan diabetic rats (the calculated P_K was 2.6 4.0 and 7.0 cm s⁻¹ 10⁻⁶ respectively) and a moderate increase compared to the ordinary very small delayed currents was found in other alloxan diabetic rats. At present these changes can not be attributed with certainty to the diabetic condition as such as alloxan has toxic effects on other organs (e.g. kidneys), which may have contributed It was concluded that (*i*) the normally low delayed P_K can, under certain (pathological) conditions, increase to very large values, (*ii*) a diffusion barrier limited the K transport from the extranodal space, (*iii*) due to the accumulation of K, action potentials were followed by an afterdepolarisation which may inactivate the P_{Na} mechanism and thereby decrease the excitability of the nerve

The present findings may be explained by the following hypothesis. Large delayed K currents may have passed through the nodal membrane of normal fibres but were not found at potential clamp because of a shunt through the Schwann cell The mechanism for this postulated K current shunt in the normal rat fibre would thus be similar to the attenuation artifact at potential clamp of (frog) myelinated nerve fibres (Dodge and Frankenhaeuser 1958), but would be selective for the K-currents if the K permeable channels are restricted to certain parts of the nodal area. Experimental evidence for such a K pathway from axon to Schwann cell has been found in the squid (Villegas 1972) If the Schwann cell-node contacts are disrupted in the alloxan diabetic rat nerve, as has been described in electron micrographs of early diphteric demyelination (Allt and Cavanagh 1969), the Schwann cell shunt would be lost This could result in the large K currents and the K accumulation found in the alloxan diabetic rat.

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On the innervation of the ileal mucosa in the rat—a synapse

By

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[illegible] classical concept of the innervation of the small intestine was that preganglionic excita[illegible] cholinergic nerve fibres and postganglionic inhibitory adrenergic fibres synapse on [illegible] esterase-positive intramural ganglion cells, thus modulating the activity of the final [com]mon pathway to the smooth muscle cell. No intrinsic adrenergic neurons have been [demon]strated in this part of the gastrointestinal tract, but extrinsic adrenergic fibres [in]nervate directly the intramural blood vessels (*cf* Furness and Costa 1971) The mere [exist]ence of efferent adrenergic and peptidergic vagal pathways to the gut (*cf* Lundberg [et a]l 1979) as well as purinergic and/or peptidergic intramural neurons (Burnstock 1972, [Sch]ultzberg *et al.* 1977) has made the original dualistic concept of gut innervation far more [com]plex.

[illegible] direct synaptic contact between nerve terminals and the gut epithelium has not yet [bee]n convincingly demonstrated at the ultrastructural level. The effects of adrenergic [agen]ts on mucosal secretion have been explained to be mediated via the vascular bed, *i.e* [adren]aline and noradrenaline both reduce mucosal blood flow and thereby inhibit gastric [aci]d secretion. However isoprenaline inhibits acid secretion independently of mucosal blood [flow,] indicating separate adrenergic actions on vessels and secretory epithelium (Curwain [and] Holton 1973). The purpose of the present study was to further investigat the possibility [of] a direct innervation of the gut epithelial cells.

[M]ale rats 150 to 200 g were decapitated. Small mucosal specimens were prepared from [the] terminal ileum. The specimens were fixed in 4% buffered glutaraldehyde, postfixed in [illegible] OsO_4 dehydrated and embedded in Araldit Ultrathin sections (600 Å) stained with [lea]d citrate and uranyl acetate were observed under a transmission electron microscope [(E]MU 4 RCA).

The mucosal glands were basally surrounded by nerve processes, frequently enclosed in [Sch]wann cell sheaths. By tracing these axons in consecutive sections towards their terminal [port]ions, a membrane to membrane contact between the nonterminal axon or the terminal [ax]el and the gut epithelial cells was often found. This is well illustrated in Fig. 1 where [illegible] axons in a bundle of nonterminal axons make direct contacts with a capillary as well [as wi]th the basal membrane of the intestinocytes. In a few cases direct contact between [ner]ve terminals and basal granulated cells with a true synaptic specialization was seen

[Key w]ords: Nerve terminal, basal granulated cell, rat ileal mucosa, synapse

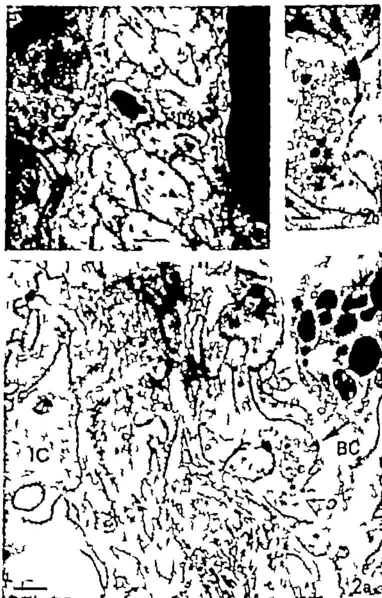


Fig. 1 From the rat ileal mucosa A thick bundle of crossectioned nonterminal axons is present between intestinocytes (IC) to the left and a capillary to the right (RBC = red blood cell). The endothelium of the capillary and the basement membrane of IC can be seen in close apposition to the axons. Bar represents 185 nm.

Fig. 2 a. Between two crypts of the rat ileal mucosa close to a basalgranulated cell (BC) to the right two nerve terminal varicosities (small arrows) can be seen. The lower one has a true synaptic contact with the BC (large arrow). Both large dense core vesicles and small round clear vesicles are present in the varicosity Bar represents 400 nm.

Fig. 2 b. Magnification of the synaptic area in Fig. 2 a. Bar represents 200 nm.

(Fig. 2). The synapsing nerve terminal contained both large dense cored vesicles (about 80 nm) and small (40–50 nm) round empty vesicles, probably representing an adrenergic terminal. However since no particular treatment for the identification of adrenergic terminals was made this terminal may be either cholinergic, adrenergic or possibly of a third type

[illegible]has earlier been stated that peripheral autonomic neurons can exert their action on [illegible]or cells by transmitter release even without true synapses, provided that the distance [illegible]e "autonomic gap" does not exceed 100 nm (Burnstock and Costa 1975). Ultrastruc[illegible] studies of the gut and pancreas have revealed such close relationships between different [illegible]s of neurons and various endocrine cells, e.g. islet cells (Kobayashi and Fujita 1969), [illegible]ochromaffin cells (Lundberg et al. 1978) and basalgranulated cells of the fetal [illegible]enum (Osaka and Kobayashi 1976). However, in this study we have observed a true [illegible]se between an autonomic nerve terminal and a basalgranulated epithelial cell. This [illegible]ates that such contacts may occur also elsewhere i[illegible] the gastrointestinal tract, not only [illegible] part of neuroendocrine complexes, but also between nerve terminals and other types of [illegible] dial cells. The presence of synapses on gut endocrine cells may indicate a nervous [illegible]ol of the release of secretory products from these cells (cf. Ahlman 1976). From the [illegible]nt study it also seems evident that axons from the same axonal bundle can directly [illegible]ate both the gut epithelium and its vasculature.

[illegible] study was supported by grants from the Veterans Administration West Side Hospital and the C. T. [illegible] Foundation and from the Swedish Medical Research Council (17X 5220, 14X 2207, 04P-4173).

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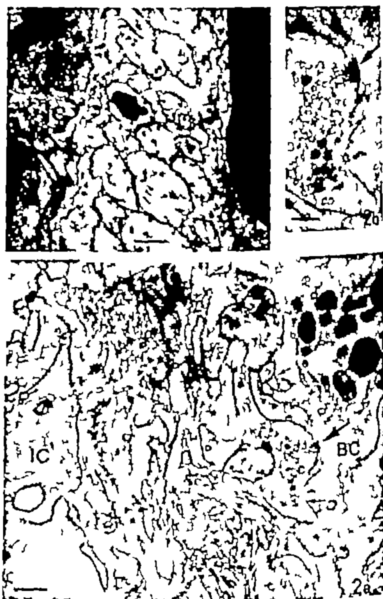


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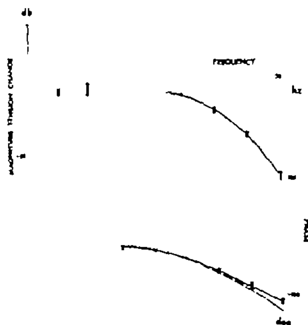


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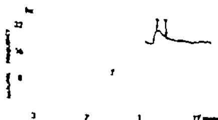
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Modulation of the isometric tension of typical unit as function of modulating frequency. The ... of the graph describes the magnitude of tension changes ... hile the bottom describes the phase ... peaks in tension and peaks in stimulus rate. The solid lines are drawn through the means of ... data ... th the bars representing the variation in six consecutive cycles. The thin dashed ... the magnitude and phase of linear critically damped second order model ... th natural ... of 7.8 Hz. The thick dashed line in phase represents the effect of an added 7 ms delay to the phase ... model.

... Beyond 2 Hz there is a rapid and progressive decrease in the tension change that ... for the same change in stimulus rate. The magnitude was graphically fitted to a ... second order system which in the case of this particular unit had a natural ... ency of 7.8 Hz. This parameter describes the point at which straight lines fitted to low ... high frequency data points intersect.

... he phase lag or delay with which tension changes follow changes in stimulus rate is ... ribed in the lower portion of Fig. 1. The phase predicted by a second order system is



2. The natural frequencies of 12 units ... ith twitch responses in relation to their twitch properties. ... rise to peak tension, □ half-decay time, ● average of rise time and half decay time. Inset: typical ... response.

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From the Otolaryngology Research Laboratory Royal Victoria Hospital, Montreal, Quebec, Canada
and the Department of Ophthalmology University Hospital, London, Ontario, Canada
and the Department of Ophthalmology University Hospital, Linköping, Sweden

Dynamics of isometric tension in single motor units in the inferior oblique muscle of the cat

By

T VILIS and G LENNERSTRAND

The extraocular muscles of the cat are unique in that the muscles are composed of the distinct types of motor units singly innervated units (SI) multiply innervated conducting units (MIC) and multiply innervated nonconducting units (MINC) (Lennerstrand 197[illegible] Previously the isometric mechanical properties of these units have been categorized in terms of twitch response fusion frequency and rate of tension rise (Lennerstrand 197[illegible] An additional method of analysing their isometric mechanical properties is the frequency response. This describes the muscle fiber s ability to generate both increases and decreases in isometric tension in response to sinusoidally increasing and decreasing stimulus rate. We found that all three types of units could most simply be characterized in terms of a linear overdamped second order system similar to that used to describe fibers in the cat soleus (Mannard & Stein 1973)

Experiments were done adult cats anesthetized with pentobarbital. Preparation of functionally [illegible] motor units in the inferior oblique muscle method of electrical stimulation, and method of recording tension is described in detail by Lennerstrand (1974).

The term stimulus rate or simply rate, will be used in this paper t describe the number of electrical pulses per second del vered t the muscle nerve. The modulating frequency will describe the period length n time, between two peaks n the sinusoidal modulation of the stimulus rate. The difference between the minimum and maximum rate will be called the mod lation depth and their average the carrier or mean rate.

Fourier analysis was performed on muscle tension using a PDP11 computer to obtain (1) an estimate of the magnitude of tension change produced n response t changes in stimulus rate and (2) the degree of phase lag with which changes in tension f llowed changes in stimulus rate

14 single motor units were fully studied These, on the basis of their isometric tension properties (Lennerstrand 1974) were subdivided into three types. 9 units had twitch contraction times shorter than 7 ms and fusion frequencies greater than 200 Hz (range of SI units). 3 units showed contraction times above 5 ms and had fusion frequencies between 90 and 175 Hz (range of MIC units) The remaining 2 units did not exhibit twitch responses while tetanic tension fused at stimulus rates below 75 Hz (properties of MINC units).

The frequency response typical of all units, is shown in Fig. 1 The amplitude of the change in isometric tension is fairly constant for modulating [illegible] between 0.25 and [illegible]

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[illegible] the Research Department, Pharmacia AB, Uppsala, the Department of Pharmacology, Royal [illegible] of Surgeons, London, and the Department of Clinical Physiology, University Hospital, Uppsala, Sweden

Anaphylatoxin-induced shock and two patterns of anaphylactic shock: Hemodynamics and mediators*

By

K. PAVEK, PRISCILLA J. PIPER and G. SMEDEGÅRD

Received 4 July 1978

Abstract

[illegible] K., P. J. PIPER and G. SMEDEGÅRD. *Anaphylatoxin-induced shock and two patterns of anaphylactic shock. Hemodynamics and mediators*, Acta physiol. scand. 1979. *105.* 393–403.

[illegible] different cardiorespiratory reactions were identified in two types of anaphylactic shock and in AT (anaphylatoxin)-induced shock. All three types had in common [illegible] portal blood pooling with [illegible] decrease in the venous return, cardiac output, and arterial pressure. In anaphylaxis (a) of the [illegible] at [illegible] low titer of hemagglutinating antibodies, the latent period was 68 [illegible] and heart and lung [illegible] unchanged. In the second type at high titer the latency [illegible] 19 [illegible] and pulmonary hypertension and decreased heart contractility occurred. After AT injection pulmonary hypertension appeared [illegible] tachypnea and unchanged heart function. Tachyphylaxis, but not cross-over tachyphylaxis against anaphylactic agent and AT [illegible] as observed in dogs and isolated guinea pig lungs. AT induced [illegible] tran[illegible] release and a [illegible] prolonged release of histamine, prostaglandins (PGs), and thromboxane A_2 and [illegible] from guinea pig lungs. SRS-A was released only in a. Indomethacin inhibited AT-induced [illegible] of PGs in guinea-pig lungs and AT-induced hypotension in the dog though it did not prevent the [illegible] in cardiac output. These model studies suggest that different patterns of clinical [illegible] can occur depending [illegible] the type of antibodies and/or mediators involved.

[illegible] C5a-anaphylatoxin, histamine, prostaglandins, SRS-A, clinical forms of anaphylaxis

[illegible] little is known about the hemodynamics of the cardiovascular shock which is occa[illegible]ally prominent in human systemic anaphylaxis (Kelly and Patterson 1974). In a [illegible] model of aggregate anaphylaxis the involvement of the heart, the pulmonary and [illegible] circulation was studied (Pavek 1977) but it was also interesting to establish [illegible] the cardiovascular shock has a single, uniform pattern or whether there are several [illegible] of cardiovascular involvements depending perhaps on the type of antibodies and/or [illegible] of mediators involved. [illegible] this study we were able to identify different patterns of [illegible] shock in two dog models of anaphylaxis, and in shock induced by C5a hog [illegible] (AT), a product of complement activation. W[illegible] were also able to identify

* [illegible] form of this report appeared as [illegible] poster at the 1975 European Immunology Meeting, [illegible]

shown as a thin dashed line If in addition, one introduces a delay of 7 ms which could be accounted for by delays introduced by the sinusoidal variation of the stimulus rate and by conduction and synaptic delays, the theoretical curve, thick dashed line, provides a good fit to the experimental data.

The range of natural frequencies for units exhibiting twitch tension (SI units and MIC units) is given in Fig. 2. It shows not surprisingly that units that have slow contraction times generally exhibit low natural frequencies The lowest natural frequencies, 4 Hz, were exhibited by the two units which did not show a twitch tension (not shown in Fig. 2. Units which exhibited twitch tension responded faster that is, their natural frequency increased if the magnitude of the modulation depth was increased. In the two units with no twitch response increase in the modulation depth produced a decrease in the natural frequency

It has been suggested (Mannard & Stein 1973) that close fit of the muscle frequency response data to a second order system underlies the fact that there are predominantly two rate-limiting processes in the modulation of isometric muscle tension, the process of binding between actin and myosin and the process involved in the decay of the active state The fact that all three types of motor units in the extraocular muscles have frequency responses which fit the same model suggests that modulation of tension here is also limited by the same process. It is not unexpected that the natural frequencies, especially of units thought to be SI are generally higher than those found in the soleous or gastrocnemius muscles (Mannard & Stein 1973 Partridge 1965) It was noted however in eleven of the twelve units that exhibited twitch tensions, that decay of tension was slower than rise (Fig. 3 suggesting that the process preventing the natural frequencies from being higher still was the decay of the active state.

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Anaphylatoxin-induced shock and two patterns of anaphylactic shock. Hemodynamics and mediators*

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In the dog, different cardiorespiratory reactions were identified in two types of anaphylactic shock and in AT (anaphylatoxin)-induced shock. All three types had in common portal blood pooling with subsequent decrease in the venous return, cardiac output, and arterial pressure. In anaphylaxis (a) of the first type, at low titer of homocytotropic antibodies, the latent period was 60 s and heart and lung function was unchanged. In the second type, at high titer, the latency was 19 s and pulmonary hypertension and decreased heart contractility occurred. After AT injection pulmonary hypertension appeared within seconds and unchanged heart function. Tachyphylaxis, but not cross-over tachyphylaxis against anaphylactic agent and AT was observed in dogs and isolated guinea pig lungs. AT induced transient release and prolonged release of histamine, prostaglandins (PGs), and thromboxane A_2 and metabolites from guinea pig lungs. SRS-A was released only in a. Indomethacin inhibited AT-induced release of PGs in guinea-pig lungs and AT-induced hypotension in the dog though it did not prevent the fall in cardiac output. These model studies suggest that different patterns of clinical [illegible] can occur depending on the type of antibodies and/or mediators involved.

Key words: C5a-anaphylatoxin, histamine, prostaglandins, SRS-A, clinical forms of anaphylaxis

Very little is known about the hemodynamics of the cardiovascular shock which is occasionally prominent in human systemic anaphylaxis (Kelly and Patterson 1974). In a previous model of aggregate anaphylaxis the involvement of the heart, the pulmonary and peripheral circulation was studied (Pavek 1977) but it was also interesting to establish whether the cardiovascular shock has a single, uniform pattern or whether there are several types of cardiovascular involvements depending perhaps on the type of antibodies and/or type of mediators involved. In this study we were able to identify different patterns of cardiovascular shock in two dog models of anaphylaxis, and in shock induced by C5a hog anaphylatoxin (AT), a product of complement activation. We were also able to identify

* An abstracted form of this report appeared as poster at the 1975 European Immunology Meeting, Amsterdam.

differences in mediators released during anaphylaxis after injection of AT into isolated guinea pig lungs. The release of mediators after injection of AT was also studied in the intact dog.

Methods

Isolated perfused guinea pig lungs

The lungs were removed from male guinea pigs (300–400 g) and perfused with Krebs bicarbonate solution via the pulmonary artery. Lungs from either normal or ovalbumin pre-sensitized guinea pigs were used. The effluent from the lungs superfused a series of assay tissues to detect the mediators of anaphylaxis. These were strips of cat terminal ileum (CTI) to detect histamine; rabbit aorta (RbA) to detect rabbit aorta contracting substance (RCS) now known to be a mixture of thromboxane A_2 and prostaglandins G_2 and H_2 (Hamberg et al. 1975); longitudinal smooth muscle of guinea pig ileum to detect slow-reacting substance of anaphylaxis (SRS-A) and a rat stomach strip (RSS) and colon (RC) and chick rectum (CR) to detect prostaglandins (PGs). All assay tissues except CTI were made more specific for RCS, SRS-A, and PGs by continuous infusion of antagonists to acetylcholine, 5-hydroxytryptamine, histamine, and catecholamines (Piper and Vane, 1969).

Identification of prostaglandins

Effluent from lungs was collected either directly from the cut heart or after superfusion over the assay tissues. Samples were collected immediately after infusion of AT into the pulmonary artery, and also from untreated control lungs. The effluent was acidified to pH 3 and extracted twice with ethyl acetate, evaporated to dryness, and subjected to thin layer chromatography in the AI system (Gréen and Samuelsson 1964).

Detection of substances released into the circulation of the dog

Dogs were anesthetized with pentobarbital sodium 30 mg/kg and prepared for use in the blood bathed organ technique (Vane 1969). An endotracheal tube was inserted to facilitate artificial respiration; after administration of heparin 1 000 u/kg b.wt., blood was pumped at 10 ml/min, passed through a dialysis machine (Collier et al. 1973), and returned to the opposite femoral vein. The substances present in the dialysate were estimated by the assay tissues as previously described.

Hemodynamics in anaphylaxis and anaphylatoxin-induced shock

In the A group 5 dogs were sensitized to horse serum by an i.v. dose of 20 ml followed by 3 i.m. injections of 5 ml over the following 3 days. After 3 to 4 weeks, a shock was elicited by challenge with right atrial injection of 10 ml horse serum.

In the B group, 7 dogs were sensitized by 20 ml of horse serum given i.v. and later at 2–3 weeks interval with 1 + 1 ml of horse serum with Freund's adjuvant given i.m. In the first booster dose, complete Freund's adjuvant was used, while in the second, incomplete Freund's adjuvant was used since repetitive administration of complete adjuvant was not tolerated locally. The dogs were challenged by right atrial injection of 10 ml horse serum one week after the last booster dose. In some of the sensitized dogs, antihorse serum antibody titers were measured using passive hemagglutination. Blood sample was taken before the challenge.

In the third group (C group) of 12 dogs AT was injected. AT (C5a) was isolated from yeast-activated hog serum using described procedure (Lefflander et al. 1972). 24 mg of AT were obtained from 36 liters of serum. Purity of AT was tested by polyacrylamid electrophoresis and 1 strong and 1 weak band were detected. Two dogs received indomethacin premedication, 3 mg/kg given i.v. 10 min before the injection of AT.

Shock experiments in dogs were performed under pentobarbital anesthesia. Respiration was spontaneous and secured by a cuffed tracheal tube. After dissection of appropriate vessels, catheters were placed into desired sites partly under X-ray control. Vascular pressures in the portal vein, right and left atria, pulmonary artery and distal aorta were measured using catheters and Statham transducers. Cardiac output was estimated by left-sided thermodilution technique: curves were detected in the aortic arch and a digital value of cardiac output appeared shortly after the injection of cold saline on a display of a cardiac output computer (Pavek 1974). Pressures and thermodilution curves were recorded on a UV recorder (Atlas Copco). In some of the experiments ECG was also recorded and hemoglobin concentration and O_2 saturation were measured in blood sampled from both systemic and pulmonary arteries (CO Oximeter, Instrumentation Laboratories).

Table I. C5a-anaphylatoxin-induced release of mediators from isolated perfused guinea pig lungs.
IA—into pulmonary artery DIR—directly to assay tissues.

	Duration of infusion	Indomethacin 1 μg/ml infusion	Histamine ng/ml	RCS	PGs ng/ml E_2	PGs ng/ml $F_{2\alpha}$	Increase in Pulmonary artery pressure, mmHg	Comments
[illegible]	Injection	—	500	+	10	5		(*)
[illegible]	Injection	DIR	500	+	10	10–20		
[illegible]	Injection	IA	500	−	<2	0		
[illegible]	2 min	DIR	500	+	20	>20		
[illegible]	2 min	DIR	>500	+	20	50		
[illegible]	2 min	IA	—	−	0			
[illegible]	2 min	DIR	500	+	10	<10	50–80	
[illegible]	2 min	DIR	1 000	+	20	<20	30–50	
[illegible]	2 min	IA	100	−	1	0	35–55	
[illegible]	2 min	DIR	1 000	+	20	20	Prolonged rise of about 10	
[illegible]	6 min	DIR	1 000	+	10	20	Very small	(*)

AT no effect DIR.
AT 6 min DIR. No effect. Only histamine release slightly longer than with 2 min IA infusion.

Results

Substance released from isolated lungs

...ykfio (1972) showed that raw anaphylatoxin from rat serum released histamine-like and ...staglandin-like material from isolated perfused lungs. Our results with pure hog C5a-AT ...firm and extend these findings (Table I). AT had no direct effects on the assay ...ues but when AT 10 μg was injected or 1–2 μg/ml infused for 2 min into the pulmonary ...ery of guinea pig isolated lungs, all assay tissues except guinea pig ileum contracted ...expts.). These results showed the release of histamine, RCS and a PG-like substance ...t no release of SRS. In experiments where AT was infused into lungs from sensitized ...nea pigs, challenge of the lungs produced similar contractions of the assay tissues. The ...ounts of histamine released, as estimated by contractions of CTI, were 0.5–1 μg/ml ...mpared with 0.5–2 μg/ml released during anaphylactic shock, additionally SRS-A was ...eased in anaphylaxis (Piper and Vane 1969). Contractions of RSS, CR, RC indicated ...at 5–20 ng/ml of prostaglandin-like material was released (assayed in terms of PGE_2 and ...), whereas 20–100 ng/ml (assayed as PG E_2) were released in anaphylaxis (Piper and ...ne 1969). Extraction and thinlayer chromatography of effluent from lungs during ...fusion of AT showed that PGs of E and F-type were released.

It was not possible to quantitate the release of RCS owing to the instability of the sub...tance but AT released an RCS which was indistinguishable from that released by anaphy...laxis. When AT was infused over 6–10 min, the release of histamine, RCS and PGs was not ...olonged. This shows that AT belongs to the group of substances which cause a short-...lasting release of histamine, PGs and RCS (Piper 1974).

In 3 expts. indomethacin 1 μg/ml was infused into the lungs before AT the subsequent ...lease of RCS was abolished and that of PGs either abolished or very much reduced

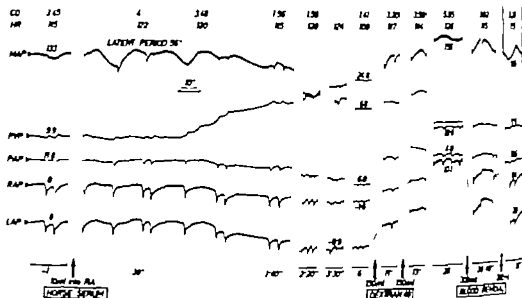


Fig. 1 Type A anaphylactic shock, dog no 49 CO—cardiac output, HR—heart rate, pressures: MP—mean arterial, PVP—portal venous, PAP—pulmonary artery RAP—right atrial, LAP—left atrial.

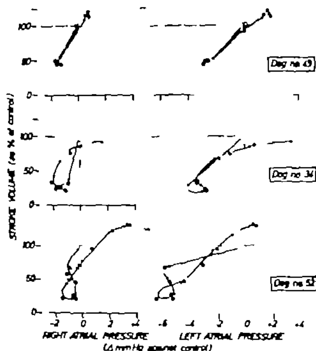
Substances released into the circulation

In 5 dogs 10 μg AT/kg was slowly injected i v There was a rapid fall in arterial blood pressure (40 mmHg) and a slow recovery over 10 min. Sometimes there was a small r[ise] of pressure at the maximum point of hypotension. Following AT injection substan[ces] contracting assay tissues entered the circulation. CTI RSS CR and RC contracted. CR a[nd] RC were blocked intraluminally with combined antagonists and their contraction show[ed] the release of PG like material This release declined slowly as the blood pressure recovered. The contractions of RSS, CR and RC were almost matched by infusions of PGs 2 5 μg infused directly over the assay tissues. The contraction of CTI suggested the release [of] histamine or kinins. Rabbit aorta showed variable responses and no definite evidence [of] RCS release.

In 3 expts. where arterial blood was dialysed after AT injection, substances contract[ing] assay tissues, like histamine and PGs, reached the assay tissues. Similar contractions [of] RSS CR, RC were produced by dialysis of blood containing PGE, 5 μg/ml dialysed blood containing histamine 2 μg/ml caused a contraction of CTI similar to that seen af[ter] injection of AT The contraction of CTO following injection of AT was terminated by sup[er]fusion of CTI with mepyramine. This strongly suggested that the contraction was due [to] histamine. Although kinins contract CTI they are not dialysed out of blood (Col[lier] *et al* 1973).

Hemodynamics

Anaphylactic shock type A This type of shock occurred in dogs sensitized only with ho[rse] serum. A typical sequence of the cardiovascular events is shown in Fig. 1 the avera[ge] changes in Fig. 4 After a challenging dose of serum there was a latent period of 68 1



2. Ventricular function curves [in type] A (dog no. 49) and type B [(dogs] no. 34 and 52) shock. Circles [indicate] values after the challenge, [and] targets are values during [and] after ... stepwise increase [in atrial] pressures by dextran

(mean ± S D) with a range from 40 to 105 s. This period was measured as time between [the a]ppearance of the cold bolus of horse serum in the aortic root as recorded by the [thermis]tor and the first distinct change in the portal venous pressure. The first detectable [ch]ange was a transient increase of cardiac output. A pronounced increase in the portal venous [pres]sure was accompanied by a small and transient (< 1 min) increase in the pulmonary [arte]ry pressure. Later the pulmonary artery pressure decreased below control level. [Ro]ughly 10 s after the beginning of the portal hypertension a decrease in both atrial [pre]ssures and cardiac output appeared followed by a drop in the systemic arterial pressure [and] sinus bradycardia. The oxygen saturation of arterial blood was unchanged, the arterio-[venous] difference in O content widened as cardiac output decreased and in the first phase [of] shock hemoglobin concentration did not indicate a consistent trend towards hemo-[con]centration. No early change in the respiratory frequency was observed with the excep-[tio]n of one case where a rapid breathing appeared (Fig. 6). However the respiratory fre-[que]ncy was always high during the developed shock.

In order to evaluate the pumping function of the heart during shock, atrial pressures were [stepw]ise increased by repeated injections of dextran in increments of 50 ml. The resulting [stro]ke volumes were plotted against the left atrial and the right atrial pressures (Fig. 2, [do]g no 49). All points during the initial shock and after dextran-induced changes lied on [or] near a thought line which crosses the initial control value. This relation was present [in] all dogs and pointed against the presence of a depressed cardiac contractility in type A [shoc]k.

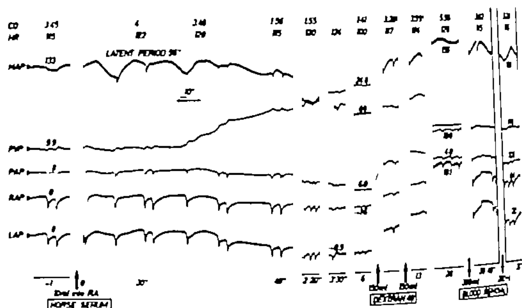


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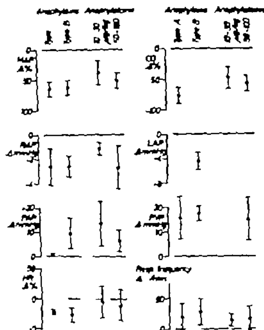
Substances released into the circulation

In 5 dogs 10 μg AT/kg was slowly injected i.v There was a rapid fall in arterial blo pressure (40 mmHg) and a slow recovery over 10 min. Sometimes there was a small r of pressure at the maximum point of hypotension. Following AT injection substanc contracting assay tissues entered the circulation. CTI RSS, CR and RC contracted. CR a RC were blocked intraluminally with combined antagonists and their contraction show the release of PG like material. This release declined slowly as the blood pressure recove The contractions of RSS CR and RC were almost matched by infusions of PGs 2-5 μg infused directly over the assay tissues. The contraction of CTI suggested the relea histamine or kinins. Rabbit aorta showed variable responses and no definite evidenc RCS release.

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Hemodynamics

Anaphylactic shock type A This type of shock occurred in dogs sensitized only with hor serum A typical sequence of the cardiovascular events is shown in Fig. 1 the avera changes in Fig. 4 After a challenging dose of serum there was a latent period of 68±1



. 4. Maximum cardiorespiratory changes ... values ... 95 confidence inter... 3 types of shock. Values till 2nd ... were taken at after AT 50–100 μg/kg; ... in other groups. Number of ... ≥5 except for LAP n 2, ... A type PVP 3 after AT 50– μg/kg

...omparison of serum levels of hemagglutinating antibodies against horse serum showed ... dogs sensitized with serum and Freund's adjuvant have a level of the order 200 times ... than dogs sensitized with serum alone.

AT-induced shock. AT was ...dministered in 9 cases into the right atrium, in 2 cases into ... left ventricle and in 1 case into a tributary of the portal vein; the ensuing reactions were ...ilar. An estimation of the dose-response showed strong reactions in the dose range ... 100 μg/kg (Fig. 4). The latent period was 17 ± 7 s and varied between 10 and 30 s.

After right atrial injections, first a pulmonary hypertension and some seconds later a ... hypertension started; while the pulmonary reaction lasted about 5 min, the portal ...action ...as sustained. The heart filling and cardiac output reacted similarly as in the ...phylaxis, heart rate ...aried and sinus rhythm was maintained. The respiratory frequency ...creased simultaneously with the pulmonary hypertension and at its height the tachypnea ...as, as a rule, directly proportional to the hypertension (Fig. 6).

After correction of the decreased right atrial pressure by dextran or autologous blood, ... cardiac output returned to its control level. Ventricular function curves showed that ... values of stroke ...olume ...ersus right atrial pressure were near a line crossing the ... control value (Fig. 5); this testified that the cardiac contractility was not changed ...ter AT injection.

The dogs premedicated by indomethacin did not show the usual hypotension after ... 30 μg/kg. The cardiac output, however, was decreased by 30–40 per cent.

Tachyphylaxis, cross-over tachyphylaxis. In 3 dogs, after recovering from anaphylactic ...(type B) horse serum was injected again, but failed to induce ... measurable response.

Fig. 3. Type B anaphylactic shock, dog no 34

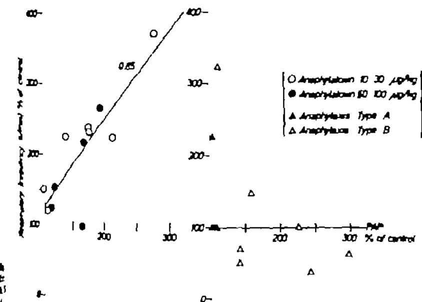
Evaluation of measurements during the shock where the low level of the right atri pressure was corrected by dextran as nearly as possible to the baseline level, showed that i those times not only cardiac output but also pulmonary artery pressure returned to th control level Also arterial pressure returned almost to the control level. Thus there wa no active change in the pulmonary vascular resistance and only a variable and sud decrease in the peripheral resistance.

Anaphylactic shock type B This type occurred in dogs sensitized with horse serum a Freund s adjuvant The latent period was short, on average 19 ± 8 s and varied betwe 10 and 30 s. Similarly as in the type A initially portal hypertension appeared follow by a decrease in atrial pressures and later by a systemic arterial hypotension. At the low cardiac output there was regularly a sinus bradycardia (Fig. 4). A pronounced pulmona hypertension appeared early reached maximum about 90 sec after challenge, and stay for 5 min or more (Fig. 3) There was no consistent hemoconcentration or decrease in the saturation of arterial blood and the arteriovenous difference in O content widened as cardi output dropped The rate of respiration initially varied in both directions, occasionally short apnea was observed Since the shock was developed, tachypnea was a rule.

Construction of ventricular function curves (Fig. 2) showed signs of right heart depressi in 6 out of 7 dogs, and of left heart depression in 5 out of 7 dogs. In 4 cases the myocard depression lasted less than 5 min and in 2 cases it was present over the whole observati period, *i.e* more than 15 min

Evaluation of the selected measurements, where the lowered right atrial pressure w corrected by dextran closely to its baseline values, showed that the pulmonary art pressure was consistently increased on the average by 75% The mean arterial press remained consistently low on the average 17% under the control value.

Thus in type B shock there was a marked and active increase in the pulmonary vasc resistance.



. Respiratory frequency at the highest level of pulmonary artery pressure in 3 types of shock.

he second of our findings is that one set of cardiorespiratory patterns was regularly found logs sensitized over a short period with the anaphylactic agent only; somewhat different erns occurred in dogs sensitized over a longer period with the agent in Freund's vant, again another set of changes was associated with the AT-induced shock.

Our findings also indicate that different mechanisms were involved in the anaphylactic AT-induced shocks. This suggestion is supported by the demonstration of a complete hyphylaxis to the anaphylactic agent and to AT in both isolated guinea pig lungs and act dogs—though under conditions of tachyphylaxis to one of the two agents, a full ht response could be sustained by the other agent. The presently observed effects AT on the pulmonary and systemic circulations are similar to findings of Bodammer in the guinea pig and cat. Bodammer concluded that these effects of AT are direct d not mediated by released histamine or prostaglandins. In our experiments histamine d prostaglandins were released after both the anaphylactic agent and AT. SRS, however s released only in the anaphylaxis. Since AT readily released histamine and prosta ... hen injected into the pulmonary circulation, it may act both directly and via lood mediators. The fact that indomethacin inhibited the AT-induced hypotension gests that some product(s) of metabolism of arachidonic acid by cyclo-oxygenase was volved in this response.

Further it appears that the titer of antibodies and the associated state of hypersensitivity the mediator and receptor levels are important determinants of the cardiorespiratory erns in the anaphylactic shock. Dogs with high titers of hemagglutinating antibodies have ring the shock a strong tendency towards a major and sustained elevation of the pulmonary ascular resistance and decreased cardiac contractility; such changes were not observed in

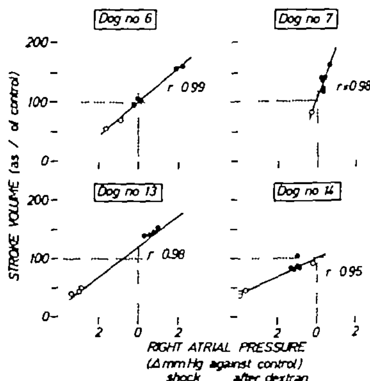
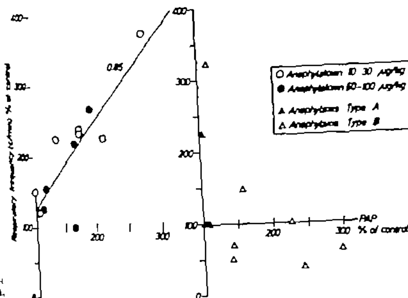


Fig. 5. Ventricular function curv after AT given in dose of 30, 3 50, 50 μg/kg in dogs no. 6, 7, 1 14

The first and second challenging injections were separate by 45 to 60 min. Similarly i dogs there was no response to the second injection of AT. In two dogs unresponsive to t second injection of horse serum, it was still possible to induce a shock by AT. Howev here the pulmonary hypertension was attenuated. Further, in two dogs unresponsive to t second injection of AT it was still possible to induce a profound anaphylactic shock (type I Thus tachyphylaxis against both horse serum and AT was observed but there was no cro over tachyphylaxis between the anaphylactic agent and AT.

Discussion

The first important finding in this report is the occurrence of a shock state with card output depressed to about 50 per cent after injections of AT in normal dogs and to abo 25 per cent after injections of the anaphylactic agent in sensitized dogs. The decrea cardiac output has obviously the same common cause in both AT induced and anaph lactic shocks: the primary portal venous blood pooling is followed by decreased veno return, decreased cardiac output and systemic arterial hypotension. This mechanis which is typical for the dog, was described in ascaris-hypersensitive dogs already in 19 by Grafia *et al.* The role of the portal pooling seems to be essential since we could prev the shock response to the anaphylactic agent injected into the right atrium in sensitized do by a balloon occlusion of the inferior vena cava and thoracic aorta.



4. Respiratory frequency at the highest level of pulmonary artery pressure in 3 types of shock.

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Our findings also indicate that different mechanisms were involved in the anaphylactic AT-induced shocks. This suggestion is supported by the demonstration of a complete hyphylaxis to the anaphylactic agent and to AT in both isolated guinea pig lungs and act dogs—though under conditions of tachyphylaxis to one of the two agents, a full ck response could be sustained by the other agent. The presently observed effects AT on the pulmonary and systemic circulations are similar to findings of Bodammer 69) in the guinea pig and cat. Bodammer concluded that these effects of AT are direct not mediated by released histamine or prostaglandins. In our experiments histamine prostaglandins were released after both the anaphylactic agent and AT. SRS, however released only in the anaphylaxis. Since AT readily released histamine and prosta- ndins when injected into the pulmonary circulation, it may act both directly and via eased mediators. The fact that indomethacin inhibited the AT-induced hypotension ggests that some product( ) of metabolism of arachidonic acid by cyclo-oxygenase was volved in this response.

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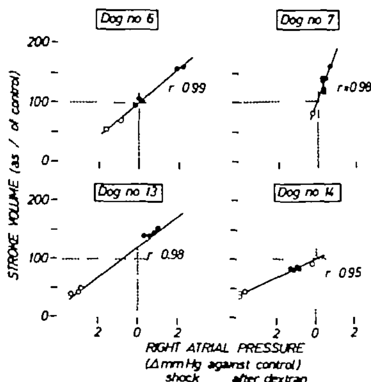


Fig. 5. Ventricular function curve after AT given in dose of [illegible], 50, 50 μg/kg in dogs no 6, 7, 13 14

The first and second challenging injections were separate by 45 to 60 min. Similarly in dogs there was no response to the second injection of AT In two dogs unresponsive to the second injection of horse serum, it was still possible to induce a shock by AT However here the pulmonary hypertension was attenuated. Further in two dogs unresponsive to the second injection of AT it was still possible to induce a profound anaphylactic shock (type II). Thus tachyphylaxis against both horse serum and AT was observed but there was no cross-over tachyphylaxis between the anaphylactic agent and AT

Discussion

The first important finding in this report is the occurrence of a shock state with cardiac output depressed to about 50 per cent after injections of AT in normal dogs and to about 25 per cent after injections of the anaphylactic agent in sensitized dogs. The decrease of cardiac output has obviously the same common cause in both AT-induced and anaphylactic shocks the primary portal venous blood pooling is followed by decreased venous return, decreased cardiac output and systemic arterial hypotension. This mechanism, which is typical for the dog, was described in ascaris-hypersensitive dogs already in 19[illegible] by Graña *et al* The role of the portal pooling seems to be essential since we could prevent the shock response to the anaphylactic agent injected into the right atrium in sensitized dogs by a balloon occlusion of the inferior vena cava and thoracic aorta.

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dogs with low titers of antibodies. This may be related to several published findings The anaphylactic process can be started via several immunological pathways (Tou and Raffel 1975) and the time period between immunization and challenge can also be important for example a moderate type of degranulation of mast cells of mice occurs during the first 4 weeks whereas an explosive type of degranulation predominates during 5 to 8 weeks (Barnett and Justus 1975) Different mediators may be responsible for anaphylactic changes at different times from the start of sensitization. Flurbiprofen, an antagonist of SRS-A induced contraction of guinea pig tracheal chain protected guinea pigs against aerosol-induced anaphylactic shock during 3–4 weeks after sensitization but was ineffective during 5–8 weeks after sensitization (Greig and Griffin 1975) The sensitivity of the smooth muscle to mediators may vary considerably according to the immunological status for instance toxicity of bradykinin is much higher in rats sensitized with horse serum and Bordetella pertussis vaccine than in controls. The vaccine itself had a weaker and more transient effect (Dawson and West 1965)

The depressed ventricular function observed during the shock only in dogs sensitized with horse serum and Freund s adjuvant can be explained partly by the loading effect of the pulmonary vasoconstriction the major cause of the left-sided failure, however appears to be a direct affection of the heart by the anaphylactic process. One mediator deserves a consideration as a causative agent is SRS-A This substance is released when antigen reacts with IgGa antibodies (Orange and Moore 1976) It is biologically active in sub nanogram quantities although its effects on the circulation have not been well documented However when SRS from cat paw (Strandberg and Ulvnäs 1971) (which is very similar to SRS-A) was infused into the isolated perfused guinea pig heart it induced both coronary constriction and depression of contractility (Bernauer W and Strandberg, K., personal communications about pilot experiments) and SRS-A may act similarly

The kind help o advice of P Borwell, H Hedin and O Tangen in connection with the preparation of anaphylatoxin is much appreciated.

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[illegible] references see Ågren and Ronquist 1976). These involved either the endogenous protein [illegible] and an extrinsic acceptor or cases where both the enzyme and the acceptor were [illegible]genous. Few of these protein kinase—mediated reactions have been cyclic AMP in[illegible]ndent (Andrew *et al.* 1973 Bacalao and Rieber 1973 Ho *et al.* 1975 Steiner 1975, [illegible] *et al.* 1975, Prasad *et al.* 1976). This includes the plasma membrane phosphorylation [illegible] for the Ehrlich, glia and glioma cells (Ågren and Ronquist 1974 Ronquist and [illegible] 1974 a, 1974 b, Ågren and Ronquist 1976) as well as 3T3 cells, both normal and [illegible]-transformed (Mastro and Rozengurt 1976).

[illegible] cyclic AMP-dependent protein kinases are known to be activated by more than one [illegible] cyclic nucleotide. Cyclic GMP-specific kinases have in recent years been found in pig (Kuo *et al.* 1976), guinea pig fetal lung (Nakazawa and Sano 1975), bovine adrenal [illegible] (Shoma *et al.* 1974), rat pancreas (Leemput *et al.* 1973) and cerebellum (Hofman [illegible] Sold 1972), mammalian smooth muscle (Casnellie and Greengard 1974) and heart [illegible] (Kuo *et al.* 1972, 1976). Smooth muscle is the only tissue in which any endogenous [illegible]strate proteins for cyclic GMP-dependent protein kinases have so far been found (Green-[illegible] 1976)

[illegible]yclic GMP-stimulated protein kinase differs from that stimulated by cyclic AMP in [illegible] to some physicochemical properties (Nakazawa and Sano 1975 Nishiyama *et al.* [illegible]5, Takai *et al.* 1975). Nevertheless, enzymatic properties for both types of protein kinases [illegible] the same in respect to the requirements for the binding steps at the 3 different sites in [illegible] molecule, namely the binding of cyclic nucleotide for activation, the binding of ATP [illegible]valent cations and the binding of protein substrate for the reaction (Kobayashi and [illegible] 1976).

[illegible] he aim of the present study was to further characterize the endogenous protein kinase [illegible] endogenous acceptor(s) of the plasma membrane vesicles obtained by the further puri-[illegible]tion by Dextran T 150 gradient centrifugation of a membrane preparation (Ronquist [illegible] Christensen 1973) from Ehrlich cells.

Materials and methods

[illegible]tran 150 was obtained by courtesy of Dr B. Ingelman, Pharmacia Fine Chemicals AB, Uppsala. Adeno-[illegible] ATP (sodium salt), dibutyryl cyclic AMP and GMP [illegible]ere purchased from Sigma Chemical [illegible]pany, St. Louis, Mo. (γ-³²P)ATP was from NEN Chemicals, GmbH, Frankfurt-on-Main, W. Ger[illegible] The ingredients of the KRB buffer [illegible]ere products from E. Merck AG, Darmstadt, Germany. All [illegible] were of analytical grade. Human albumin (No. 50067) was from KABI AB, Stockholm, Sweden. *[illegible] of tumour cells and plasma membrane fraction.* The Ehrlich ascites tumour cells were grow[illegible] 7-8 days in 5-week-old male Swiss albino mice obtained from the Anticimex Breeding Farm, Norr[illegible], Stockholm. The tumour cells [illegible]ere separated by centrifugation of the ascites fluid, [illegible]hich had been [illegible] without delay 10-fold [illegible]ith ice-cold Krebs-Ringer bicarbonate (KRB) medium in order to diminish [illegible] tendency of cell [illegible]gglutination. The cells were washed twice in the KRB-medium. Final [illegible] was [illegible] in [illegible] sucrose (0.250 M)-tris-glycyl-glycine (0.016 M) buffer pH 8.0, also containing 2 mM [illegible] followed by centrifugation. This [illegible]as carried out in the International Refrigerated Centrifuge by [illegible] for 10-15 [illegible] up to at most 3 700 rpm followed by retardation for about 2 min. The membrane [illegible] preparatory procedure of Ronquist and Christensen (1973) was followed exactly.

[illegible] further purification of the membrane fraction was achieved by applying membrane material corre-[illegible] to about 45 mg protein in [illegible] Dextran T 150 gradient. The gradient [illegible]as composed by mixing equal [illegible] of [illegible] sodium phosphate-magnesium-buffer and the same buffer containing 19 % w/v of Dextran

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Phosphorylation of endogenous proteins by endogenous protein kinase of density gradient—purified plasma membrane vesicles of Ehrlich cells

By

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Received 3 August 1978

Abstract

RONQUIST G G ÅGREN and I LINDQVIST *Phosphorylation of endogenous proteins by endogenous protein kinase of density gradient—purified plasma membrane vesicles of Ehrlich cells* Acta physiol scand 1979 *105* 404–413

A phosphorylatio of endogenous acceptor protein( ) has been demonstrated to occur in membrane vesicles prepared from Ehrlich ascites tumour cells. The reaction was catalyzed by endogenous protein kinase in the presence of exogenous (γ-^{32}P)ATP A considerable increase of the specific protein kinase activity took place when th plasma membrane preparation was subjected to a further gradient centrifugation in De tran T 150. This was done in the presence of a slightly alkaline phosphate buffer containing Mg-ions which resulted in the formation of a well defined vesicular preparation at density 1.03 in the gradient The apparent K_m and V_{max} for the reaction with vesicles and exogenous (γ-^{32}P)ATP were determined and found to be 0.022 mM and 0.23 nmol mg^{-} 10 min^{-1} respectively. Neither cyclic AMP nor cyclic GMP did stimulat the protei k nase-catalyzed reaction. Instead, a clear inhibition of the reaction by the cyclic nucleotides was unexpectedly registered. Adenosine at 0.5 mM also inhibited the reaction. Calcium ions were inhibitory at all concentrations tested I the presence of a fixed (γ-^{32}P)ATP/Mg^{2+} ratio. When Mg-ions were stoichiometrically replaced by Ca-ions practically no activity was observed.

The presence of endogenous protein kinase at the surface of Ehrlich cells catalyzing the phosphorylation of endogenous membrane protein(s) was first described 1970 (Ågren and Ronquist 1970 Ronquist and Ågren 1970) and other reports followed (Ågren and Ronquist 1971 Ronquist and Ågren 1974 a 1974 b Ågren and Ronquist 1976, Ronquist et al. 197[illegible]. Schlatz and Marinetti (1971) described the cyclic AMP dependent phosphorylation of isolated rat liver plasma membrane by a partially purified protein kinase from rat [illegible] In a similar experiment Kinzel and Mueller (1973) were able to demonstrate the phosphorylation of endogenous acceptor proteins at the surface of intact HeLa cells in the presence of a protein kinase isolated from rat skeletal muscle and (γ-^{32}P)ATP in the external medium This reaction was also stimulated to a limited degree by cyclic AMP Protein kinase-catalyzed membrane phosphorylations have subsequently been published for many membrane systems

Table I. Protein content of Ehrlich cells and membrane subfractions. 3.6 × 10^9 Ehrlich cells were used for the preparation of plasma membrane fraction. Subsequent purification followed by gradient centrifugation of the plasma membrane fraction resulting in the recovery of membrane vesicles at density 1.03–1.04 of the gradient.

Sample	Protein content in mg	Per cent of total
Homogenate of Ehrlich cells (3.6 × 10^9)	1 053	100
Plasma membrane fraction	44.8	4.3
Membrane vesicles after gradient centrifugation	2.0	0.19

Results

Table I illustrates the protein content of a homogenate from intact Ehrlich cells. During the course of the work it turned out to be most convenient to start with 3.6 × 10^9 Ehrlich cells corresponding to about 1 050 mg protein (1 ml of packed Ehrlich cells is equivalent to 5 × 10^8 cells). The protein content of the plasma membrane preparations before gradient centrifugation was 4.3% of that for the Ehrlich cells corresponding to about 23 times purification. The protein content of the vesicular material of the main component after gradient centrifugation implied another 23 times purification. The vesicular nature of the material recovered from the main component at density 1.035 in the gradient (Fig. 1) is clearly seen in Fig. 2. The vesicles vary in size (Fig. 2 a) and are bounded by a typical three-layered membrane, 7–9 nm thick (Fig. 2 b).

Table II shows a considerable increase in specific activity for membrane vesicles, i.e. (^{32}P)phosphoryl group transfer into phosphorylserine- and phosphorylthreonine-residues of membrane protein. The (^{32}P)phosphoryl group transfer reaction from exogenous (γ-^{32}P)ATP into endogenous acceptor(s) of the membrane vesicles is a relatively slow process as seen in Fig. 3, and an incubation time of 10 min still represents initial velocity under the conditions used. A Lineweaver and Burk graph for the phosphoryl group transfer reaction is shown in Fig. 4. The apparent K_m is 0.022 mM and the apparent V_{max} is 0.23 nmol · mg^{-1} · min^{-1}. 0.5 mM adenosine inhibits the transfer reaction by about 45% and this inhibition is not further increased by a higher adenosine concentration (Table III).

Table II. Protein kinase activity in plasma membrane fraction and membrane vesicles obtained by gradient centrifugation. Experiments performed in basic incubation medium containing 0.05 mM (γ-^{32}P) ATP and 4 mM Mg^{2+} for 10 min at 37°C. Values are given in pmol (^{32}P)phosphorylserine- and (^{32}P)phosphorylthreonine-residues recovered per mg of protein.

Sample	(^{32}P)phosphoryl-serine, pmol/mg	(^{32}P)phosphoryl-threonine pmol/mg
Plasma membrane fraction	1.1	0.23
Membrane vesicles obtained by gradient centrifugation	164	34.9

MAIN COMPONENT

PELLET

Fig. 1 Separation of plasma membrane material upon Dextran T 150 gradient centrifugation resulting in the recovery of a main component consisting of 3 scanty layers at density 1.03–1.04. For details, see text.

T 150. The strength of the buffer was 5 10^{-4} M as regards sodium phosphate and 1 10^{-4} M as regards magnesium sulfate The pH of the buffe was 8.0. Centrifugation proceeded for 16 h at 23 000 rpm at MSE Super speed 65 centrifuge Rotor SW 3 25 (60 000 g). A main membraneous component, consisting of 3 scanty layers was recovered at a density-interval of 1.03–1.04 of the gradient (Fig. 1). The three layers of the main component were carefully removed and suspended in 130 mM NaCl and 25 mM KCl and centrifuged for 45 min at 30 000 rpm in the MSE Super speed 65 centrifuge The pellet thus obtained was resuspended in 8 ml of 130 mM NaCl and 25 mM KCl before incubation.

Incubation procedure The basic incubation medium was the following in a final volume of 10 ml containing the isotonic vesicular suspension of 130 mM NaCl and 25 mM KCl 350 μmol Tris-acetate buffer pH 7.5 5 μmol glutathione (reduced form) 40 μmol $MgCl_2$, 10 μmol of unlabeled orthophosphate (disodi m form) usually 250–500 nmol of (γ-^{32}P)ATP (Spec. act. 0.5 μCi/nmol) The vesicular membrane material corresponded to 2–4 mg protein.

The membrane vesicles were prewarmed for 4 min at 37° in an atmosphere of 93.5 oxygen and 6.5 carbon dioxide before incubation. In the experiments where Mg-ions were replaced by Ca-ions, the latter ions were included during the 4 min prewarming step. Incubation proceeded for arious times, generally 5–10 min, and was terminated by the addition of 10 ml of 20 trichloroacetic acid (TCA). Just before TCA precipitation 50 mg of human lbumin were added t the incubation medium to co-precipitate the small mounts of membrane material. To achieve a complete precipitation the denatured material was kept fo at least 16 h at 4 C in the TCA-solution before centrifugation. The pellet was washed once with 10% TCA containing an excess of unlabeled ATP and orthophosphate.

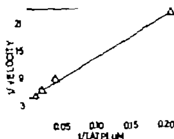
Analytical methods The washed precipitate was dried in ethanol and ther followed by partial hydrolysis according to Lipmann (1933), with 10 times the protein weight of 2 M HCl under reflux on a water bath f 20 h. The HCl was removed by repeated evaporations (4 times) *in vacuo* and the hydrolysate was mixed with 1 mg of unlabeled phosphorylserine 1.5 mg of phosphorylthreonine nd 1.5 mg of orthophosphate and applied n a 50 ml Dowex 50 column. Elution was carried out with 0.01 M HCl and labeled phosphorylserine- and phosphorylthreonine-residues were isolated and identified (Ågren and Ronquist 1970).

Radioactivity was determined in a Nuclear Chicago Scintillation Counter and (^{32}P)radioactivity determined by measuring the Cerenkov radiation.

Prot in was determined according t Lowry *et al.* (1951) with bovine serum albumin as standard.

Electron microscopy The main component at density 1.035 in the gradient usually consisting of 3 layers was collected by withdrawing 2 ml fractions. Each fraction was resuspended in excess of the sodium phosphate-magnesium-buffer pH 8.0, nd washed twice Each washing was performed by centrifugation at a Spinco L50 centrifuge rotor 40 f r 10 min at 20 000 rpm. The washing procedure was followed by fixation in glutaraldehyde (2.5% glutaraldehyde n the sodium phosphate-magnesium-buffer) for 20 min at room temperature Washing twice followed in the sodium phosphate-magnesium-buffer and centrifugation in the Spinco centrifuge for 10 min at 20 000 rpm. The material was then fixed in OsO_4 (final concentration was 1% in the abovementioned buffe) f r 20 min. Two washings followed in this buffer

Dehydration was started with 50% ethanol solution and performed with increasing concentrations of ethanol up to 100% and followed by propylene-oxide treatment before embedding in Epon (Luft 1961). During the course of fixation and dehydration, the membranous material was transformed into a dispersed floculation, and centrifugation at low speed was necessary t each step of the procedure, because of a very slow sedimentation by gravity lone. Sections we cut stained by uranyl acetat and lead citrate (Reynolds 1963) and examined in Philips EM 301



A Lineweaver-Burk graph of the protein kinase activity measured as the recovery of (^{32}P)phosphorylserine residues, of vesicles obtained by gradient centrifugation. The (γ-^{32}P)ATP/ ratio was maintained constant. Incubation proceeded for 5 min at 37°C.

Discussion

About 4.4 per cent of the total cell protein content was recovered in the plasma membrane fraction. The subsequent gradient centrifugation in Dextran T 150 resulted in a further purification implying again a 4.4 per cent recovery calculated on a protein basis. The specific protein kinase activity of the vesicular membrane material of the main component was considerable and exceeded that of the plasma membrane fraction by more than 140 times. At least two explanations might exist. One is the homogenous vesiculation of the membrane material on gradient centrifugation in the slightly alkaline Mg^{2+}-containing buffer. It means that the prerequisite conditions were available for the same sidedness for all vesicles. Further, it is reasonable to assume that the protein kinase is at the outer surface of the vesicles provided that the vesicles are sealed and impermeable for (γ-^{32}P)ATP (*cf.* Glynn 1968, ... and Ronquist 1969). We have previously demonstrated the presence of endogenous

Table III. Effect of two different concentrations of adenosine on the protein kinase of vesicles purified by gradient centrifugation. The experiments were performed in the basic incubation medium containing 0.05 mM (γ-^{32}P)ATP and 4 mM Mg^{2+} together with 0.5 mM and 1 mM adenosine, respectively for 5 min at 37°C. Activity is expressed in pmol (^{32}P)phosphorylserine- and (^{32}P)phosphorylthreonine-residues recovered per mg protein.

Incubation No.	(^{32}P)phosphoryl-serine, pmol/mg	(^{32}P)phosphoryl-threonine, pmol/mg
Control, no adenosine added	109	28.3
I. plus 0.5 mM adenosine	61.4	16.0
I. plus 1.0 mM adenosine	64.6	9.1

Table IV. Effect of dibutyryl cyclic AMP and dibutyryl cyclic GMP on the protein kinase of vesicles purified by gradient centrifugation. The experiments were performed in the basic incubation medium containing 0.025 mM (γ-^{32}P)ATP and 2 mM Mg^{2+} together with 1 · 10^{-4} M dibutyryl cyclic AMP or 1 · 10^{-4} M dibutyryl cyclic GMP for 5 min at 37°C. Activity is expressed in pmol (^{32}P)phosphorylserine- and (^{32}P)phosphorylthreonine-residues recovered per mg protein.

Incubation No.	(^{32}P)phosphoryl-serine, pmol/mg	(^{32}P)phosphoryl-threonine, pmol/mg
Control, no cyclic nucleotide added	106.3	35.3
As I. plus 1 · 10^{-4} M cAMP	61.4	21.8
As I. plus 1 · 10^{-4} M cGMP	53.9	23.6

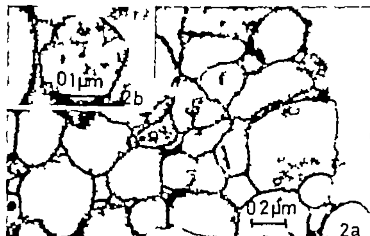


Fig 2. Electron micrographs of membrane material recovered at density 1.035 of the Dextran T gradient. Vesicles dominate (Fig 2 a) and are bounded by a 7-9 nm thick membrane (Fig 2 b, and Magnifications, 2 a 58 000, 2 b 200 000.

In accordance with previous findings on intact Ehrlich cells and a plasma membrane preparation from these cells (Ronquist and Ågren 1974 a, Ågren and Ronquist 1976, Ronquist *et al* 1977) cyclic AMP and cyclic GMP did not stimulate the protein kinase reaction in the membrane vesicles (Table IV)

Instead an unambiguous inhibition by about 40–50 per cent was achieved by the cyclic nucleotides. Table V illustrates the influence of Ca-ions on the incubation system at different concentrations. Keeping the (γ-P)ATP/Mg^{2+} concentrations constant at 0.025 mM and mM respectively increasing Ca^{2+} concentrations resulted in enhanced inhibition of the protein kinase reaction. At 5 mM Ca concentration the inhibition amounted to about per cent and when all Mg^{2+} were stoichiometrically replaced by Ca the inhibition was nearly 100% (Table V). Therefore the membrane-linked protein kinase had an absolute requirement of Mg vis-à-vis Ca

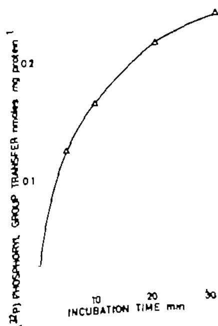
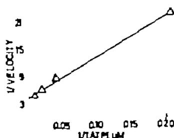


Fig. 3. Time course of protein kinase reaction, as measured by the recovery of (^{32}P)phosphorylserine residues, of vesicles obtained by gradient centrifugation. Experiments performed in basic incubation medium containing 0.05 mM (γ-^{32}P)ATP and 4 mM Mg^{2+} at 37°C.



...4. A Lineweaver-Burk graph of the protein kinase activity measured as the recovery of (^{32}P)phosphorylserine residues, of vesicles obtained by gradient centrifugation. The (γ-^{32}P)ATP/ ... was maintained constant. Incubation proceeded for ... at 37°C.

Discussion

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4.1 plus 1 · 10^{-4} M cAMP	61.4	21.8
4.1 plus 1 · 10^{-4} M cGMP	53.9	25.6

TABLE V Effect of different concentrations of Ca^{2+} on the protein kinase of vesicles purified by grad centrifugation The experiments were performed in the basic incubation medium contai 0.025 mM (γ-^{32}P)ATP and 2 mM Mg^{2+} except in experiment No. 6 where Mg^{2+} was equi metrically replaced by Ca^{2+} together with 4 different concentrations of Ca^{2+} for 5 min at 3' Activity is expressed in pmol (^{32}P)phosphorylserine- and (^{32}P)phosphorylthreonine-residues r covered per mg protein.

Experiments No.	(^{32}P)phosphoryl-serine pmol/mg	(^{32}P)phosphoryl-threonine, pmol/mg
1 Control, no Ca^{2+} added	110.3	25.7
2. As 1 plus 0.5 mM Ca	114.6	22.7
3 As 1 plus 1.5 mM Ca^{2+}	70.2	14.9
4 As 1 plus 3.0 mM Ca	34.5	7.8
5 As 1 plus 5.0 mM Ca	23.8	3.3
6. As 1., but all Mg^{2+} replaced by Ca^{2}	1.23	0.21

The membrane vesicles were pre-incubated for 4 min with the Ca-ions (2 mM).

protein kinase at the outer surface of intact Ehrlich cells as well as of glia and glioma c (Ronquist and Ågren 1974 a, 1974 b Ågren and Ronquist 1976, Ronquist *et al.* 19 Ågren and Ronquist 1978) and this observation has been confirmed by others on other types (Mastro and Rozengurt 1976, Schlaeger and Köhler 1976). This finding togetl with the fact that the vesiculation was performed under conditions that favoured right si out formation in human erythrocytes (Kant and Steck 1972) are in accordance with view that the vesicles studied are most probably right side-out contrary to the vesicles the plasma membrane preparation, that might be inside-out (Wernstedt *et al.* 1975).

Determination of possible adenyl cyclase as a marker enzyme was carried out accord to Wikström and Ågren (1973) A slight adenyl cyclase activity was registered with vesicles. This activity was increased 4 times when including Triton in the incubation medi favouring the view that the vesicles were right side out. Thus, Triton facilitated the entra of the ATP to the catalytic site of the membrane-bound enzyme system.

The other explanation could be the dissociation of a possible inhibitor to the prot kinase on gradient centrifugation or revealing of an active site of the enzyme being "cryp prior to this treatment Also the stoichiometric relationship between the protein kin and phosphoprotein-phosphatase molecules (*cf* Ågren and Ronquist 1978) present at surfaces of the intact cells and the vesicles might have changed so that proportionally m protein kinase molecules than phosphoprotein phosphatase molecules are present at surface of the vesicles. If such were the case, it also explains the time extension for maxir phosphorylation of the vesicles compared with intact cells (Ågren and Ronquist 19 In accordance with previous work cyclic nucleotides failed to stimulate the protein kin activity of the vesicles (Ronquist and Ågren 1974 a, 1974 b Ågren and Ronquist 19 Mastro and Rozengurt 1976, Ronquist *et al* 1977) Instead, a clear inhibition by both butyryl cyclic AMP and dibutyryl cyclic GMP was observed.

The aliphatic dibutyryl analogues of the cyclic nucleotides were used since they c penetrate the membranous structure. This was appropriate, since a possible effect by cyclic nucleotides could be achieved regardless of a *cis*- or *trans* position of the c

cleotide vis à vis the substrate ATP. The inhibitory effect by the dibutyryl derivatives could not be explained simply by a chelating reaction with Mg^{2+} since this divalent cation was present in a great excess. Furthermore, one might argue that the cyclic nucleotides could be hydrolyzed since no diesterase inhibitor was present in the incubation system. However, an approximate 50% reduction of the cellular content of the diesterase could be detected in the highly purified vesicles (cf. ref. Cheung 1967). Also, no inhibitory effect was to be anticipated when most of the cyclic nucleotides were hydrolyzed, since 5'-AMP at 10^{-4} M concentration in the incubation medium did not influence the reaction (unpublished observation). This finding was unexpected but in accordance with recent reports by Gatt and Kang (1977) as well as by Lucid and Griffin (1977) who studied other plasma membrane fractions and found that cyclic AMP clearly decreased the cyclic AMP independent phosphorylation of membrane proteins in their experimental systems.

Also, the inhibitory effect by 0.5 mM adenosine was congruous to the results from an experimental system of beef thyroid tissue involving a cyclic AMP dependent protein kinase (... and Field 1976).

The findings that incorporation of the (γ-^{32}P)phosphoryl group of ATP into phosphoryl serine and phosphoryl threonine is inhibited to different extent by adenosine, the dibutyryl cyclic nucleotides and Ca^{2+} suggest that more than one protein kinase is present in the membrane.

A cyclic nucleotide dependency of the protein kinase at the outer surface of the membrane does not appear to be obligatory for protein kinase being involved in a regulatory phosphorylation function. Instead, we propose an autoregulatory mechanism based upon the Ca^{2+}/Mg^{2+} ratio in the microenvironment of the membrane. An additional component of the autoregulatory mechanism might be the possible phosphoprotein phosphatase activity which is higher in the presence of Ca^{2+} than with Mg^{2+} contrary to the protein kinase activity. There is evidence that the phosphorylation of the surface membrane occurs also in vivo (Ågren and Ronquist 1978) and a transfer of phosphoryl groups can take place between cells (Ågren and Ronquist 1978). It has been claimed that exogenous ATP can influence the permeability properties of some cells (Hempling et al. 1969) and such an influence might be mediated by a phosphorylation of the surface membrane (Gazitt et al. 1976, ... et al. 1977).

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Progesterone effects on the evolution of myometrial activity in vivo in the non pregnant rabbit

By

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Abstract

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In order to study the influence of progesterone on the myometrial activity ovarian secretion of progesterone was stimulated by intra enous injection of human chorion gonadotrophin (HCG, 75 IU) in 9 nonpregnant rabbits. 4 rabbits, used as controls, were given only saline. Plasma progesterone concentration in blood samples taken at frequent intervals (2–4 h) during the recording was measured by radioimmunoassay. In the controls, the myometrial activity and the plasma levels of progesterone did not change. After HCG administration the amplitude of contraction began to decrease and the frequency began to increase after 3 h. After about 5 h the changes i amplitude and frequency were generally fully developed. This pattern of myometrial activity persisted throughout the recording period (12 h) and as also present in recordi gs made after 24 nd 48 h of HCG injection. A maximal increase in plasma progesterone concentration (5.0 ng/ml) had already occurred by 2 h after the HCG injection and gradually decreased thereafter. It is concluded that an elevation in the le el of progesterone in non-pregnant rabbits resulting from increased ovarian output (through HCG) has pronounced and immediate depressing effect on the character of myometrial activity which is indicative of a shift from nonpregnant t pregnant type.

It is well known that the pattern of myometrial activity differs between the non-pregnant and pregnant conditions in animals as well as in humans. Generally in early pregnancy the uterine activity is weaker and more uncoordinated than in non-pregnant condition. Moreover in early pregnancy the uterus is relatively sensitive to stimulants (Laudański et al. 1977). Although it has been widely speculated that progesterone is a major factor in bringing about the change in the pattern of myometrial activity from the non-pregnant to the pregnant type, there is little direct evidence showing that such an effect is in fact caused by exposure to increased concentration of progesterone (see Porter 1974). Furthermore it was suggested that there is a latency period in the action of progesterone even after an intravenous administration of 17–24 h (Schofield 1955 Csapo and Lloyd-Jacob 1961). The dose of progesterone generally used in these earlier studies was very large and far from physiological

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It has been clearly demonstrated that an exogenous administration of human chorion gonadotrophin (HCG) to rabbits causes marked and rapid changes in the ovarian steroid secretion, particularly of progestins (Hilliard 1973 Mills *et al.* 1972). We chose this approach in the present study to investigate the progesterone induced changes in the myometrial activity. Within 1 h after an i.v. injection of HCG the levels of plasma progesterone, as well as of 20α-dihydro-progesterone increase markedly (Hilliard 1973, Mills *et al.* 1972). The ovarian secretion of these steroids remains high for about 4–6 h and then declines to low levels after about 8 h, while approaching the ovulation time. An identical pattern of ovarian steroid secretion is observed after coitus-induced ovulation.

By injecting HCG during a continuous recording of the myometrial activity in rabbits while also collecting blood samples for assay of progesterone before and at frequent intervals after the HCG injection we were able to demonstrate that an elevation in the plasma progesterone level was responsible for a change in the character of myometrial activity.

Methods

Rabbits of the Swedish Land race weighing about 3 kg were used. They were non-pregnant but had all been pregnant at an earlier date. The rabbits were housed under standardized conditions of light and temperature and were fed freely with mixed diet and tap water.

The myometrial activity was recorded as changes in intrauterine pressure. The pressure sensors were inserted at laparotomy during anaesthesia with pentobarbitone sodium (Mebumalnatrium 6 .., ACO, Sweden). In 9 of the does referred to as "acute" rabbits, the intrauterine pressure recordings were performed directly following the operation (Laudański *et al.* 1977) by a micro-transducer catheter (Åkerlund *et al.* 1978). In another 5, referred to as "chronic" rabbits, the recordings were obtained at least one month after insertion of the pressure sensors. A recording system with a sponge-tipped saline-filled polyethylene catheter (Bengtsson 1968) with the tip in the middle of one uterine horn was used in this group.

The intrauterine pressure recordings were analysed for frequency (number of pressure cycles during ... min) and amplitude of the contractions. These calculations were done on 20–40 min long parts of the ... recording at various intervals. The analysis were done by the same person and only on parts of the recording without unusual artefacts (resulting from movement of the animal, pentobarbitone injection, ...). Changes in intrauterine pressure smaller than 2 mmHg were not included in the calculations.

... rabbits

In 4 "acute" does the myometrial activity was recorded for 10 h without administration of any hormone.

HCG-treated rabbits

In 5 "chronic" and 4 of the "acute" does, all with stable and regular uterine activity were given HCG, 75 IU, i.v. (Gonadex, LEO, Sweden). The myometrial activity was recorded 1–2 h before the injection and during the subsequent 12 h period. In two of the "chronic" rabbits recordings were also obtained during a period of 3 h on each of the following two days. For the determination of concentration of progesterone and oestradiol-17β in plasma (Batra *et al.* 1978) venous blood samples (2 ml) were collected from the catheterised femoral vein before and 2, 4, 8, and 12 h after the injection of HCG. In 4 of the "chronic" rabbits, samples were also obtained at 24 h after the HCG injection.

Results

After administration of hormones the uterine activity followed the typical pattern observed in non-pregnant rabbits (Laudański *et al.* 1977). The contractions occurred with a comparatively high amplitude, low frequency and long duration, and there was a distinct relaxation

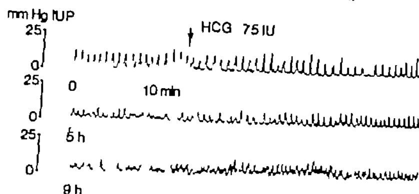


Fig. 1 Effect of HCG injection on the pattern of intrauterine pressure (IUP) recording in a non-pregnant rabbit with a chronically implanted sponge-tipped catheter

phase after each contraction (Fig. 1 2) The uterine activity in the control does did not change significantly during the recordings (not shown).

After the injection of HCG a relatively rapid change in the uterine activity occurred; it was visible in some cases as early as 1 h after the injection (not shown) and was conspicuous after 3 h of injection (Fig. 2) The uterine contractions gradually became smaller in amplitude, more frequent, more irregular and a well defined relaxation phase between the contractions could no longer be observed. This type of activity became successively more pronounced with time up to at least 9 h. There were no qualitative differences in the spontaneous activity and in the response to HCG administration between the "acute" and the chronic" rabbits (Fig. 1 2). In the two "chronic" rabbits recorded after one and two days of HCG injection the myometrial contractility was still depressed.

The plasma progesterone levels before and after the HCG injection are shown in Fig 3 A maximal increase in progesterone concentration (5 0 ng/ml) occurred already in the first sample taken at 2 h after the injection. The levels gradually decreased thereafter and the mean concentration in samples taken at 24 h after injection was 0.25 ng/ml (not shown [illegible]

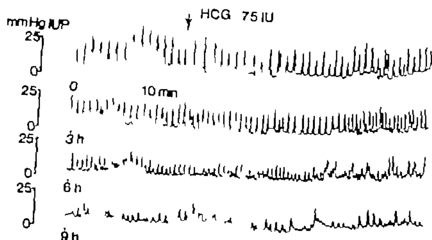
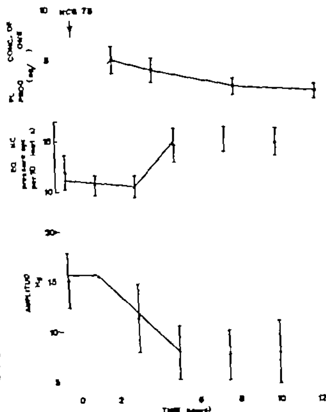


Fig. 2. Effect of HCG on the pattern of IUP in [illegible] abbit recorded by a micro-transducer catheter installed at an operation 1 h before the recording. The recording pattern and the changes caused by HCG injection were, in general, the same as in the chronic" rabbits (Fig. 1).



1 Time courses of the changes [illegible] plasma concentrations of [illegible] and amplitude and [illegible] of uterine contractions [illegible] an HCG injection. Values [illegible] mean S.E. of 7 to 9 [illegible] analyses. The S.E. of the [illegible] mean value of pro[illegible] is included within the [illegible].

[illegible] figure). The HCG injection caused no significant change in the plasma oestradiol con[illegible] which ranged between 12 to 64 pg/ml in all samples analysed.

The change in the contractile activity pattern after HCG injection seemed to follow the [illegible] in progesterone concentration in plasma (Fig. 3). The amplitude of contraction [illegible] to decrease 1 h after the HCG injection and the frequency began to increase after [illegible] 3 h. After about 5 h these changes were fully developed. This pattern of myometrial [illegible] with high frequency and low amplitude of contractions, was maintained during the [illegible] of the observation period in spite of a steady decrease in the peripheral plasma pro[illegible] concentrations following the peak value after 2 h of HCG injection.

Myometrial tissue concentration of progesterone (Batra and Bengtsson 1976) was meas[illegible] in 4 rabbits 12 h after HCG injection. The mean progesterone concentration was 15 7 [illegible] [illegible] weight (range 11.5–17 1).

Discussion

[illegible] continuously recording the rabbit myometrial activity under conditions where con[illegible] of progesterone were changing drastically but within physiological limits, we [illegible] able to delineate the effects of this hormone on the myometrial activity. At about 5 h [illegible] HCG injection a distinct change, indicative of a shift from non-pregnant to pregnant

type (Laudański *et al* 1977) in the pattern of myometrial activity was observed. The plasma progesterone concentration was already maximal at the first sampling, 2 h after HCG in- [illegible] jection and thereafter decreased gradually The peak progesterone concentration may have occurred even earlier than after 2 h but the majority of the published data indicate that the maximal plasma progesterone concentration is attained between 2 and 4 h after HCG administration (Hilliard 1973) These observations together with our recent data showing the effects of infusion of progesterone itself on the myometrial activity (Laudański *et al* 1978) support the contention that progesterone was the major factor responsible for the shift in the pattern of myometrial activity

The possibility of a direct effect of HCG on the myometrium seems unlikely since one would expect the manifestation of this not only immediately but also short-lived after a single i v injection Dessarzin and Stamm (1962) reported that the administration of HCG was able to inhibit the myometrial activity to the pregnant rat *in situ* This inhibitory effect was observed within 1 h after an intramuscular injection of HCG A comparison of the present results with those published by these authors is difficult due to the species difference and to the fact that they used pregnant animals, whereas we studied the effect in non-pregnant ones

Although the concentration of 20α-dihydroprogesterone after HCG administration shows a dramatic increase, more than 10 times that of the progesterone concentration, we are not aware of any report on the effect of this progestin on the myometrial activity *in vivo* From the present observations one could deduce at least that the effect of 20α-dihydroprogesterone could not be opposite to that of progesterone, since in that event it would have counteracted the effect of progesterone. The extent to which 20α-dihydroprogesterone might have reinforced the effect of progesterone by acting synergistically cannot be evaluated until the effects of this steroid individually and in combination with other steroids are known. Preliminary experiments indicate (unpublished) that 20 -dihydroprogesterone in related high doses had no or slight inhibitory effect on the rabbit myometrial activity

Schofield (1955) who studied rabbit myometrial activity *in situ* found no immediate effect of progesterone given i v and i.m and came to the conclusion as did Csapo and Lloyd- [illegible] Jacob (1961) and Porter (1974) that progesterone has a latency in its action on the myometrium. However these enormous doses of progesterone, together with the vehicles [illegible] could even have had a toxic effect, and interestingly Schofield (1955) reported that the rabbits often died within 30 min of injection of the vehicle, propylene glycol (see also Ka [illegible] 1977 Luukkainen and Csapo 1961)

The latency in the effect of progesterone on myometrial activity even after i.v administration reported in the literature (Schofield 1955 Csapo and Lloyd-Jacobs 1961 Porter 1974) seems somewhat paradoxical in view of the fact that steroid hormones are considered to act by altering ion permeability or/and conductance characteristics of the myometrial cell membrane (Abe 1970 Kao 1977 Batra and Bengtsson 1978) The present results showing an almost immediate effect after an elevation of progesterone in plasma together with the data demonstrating a rapid change in myometrial activity after lyteolysis in pseudopregnant rabbits (Laudański *et al* 1978) clearly contradict the theory of a latency in progesterone effect The latency might have resulted from the use of chronic type rather than the acute

[illegible] of rabbits in studies reporting this phenomenon. The inflammatory reaction, intra-[illegible] infection, adhesions etc., which frequently will occur in rabbits with chronically [illegible] catheters, could significantly affect the intramural diffusion and passage of the [illegible] hormone. However the fact that both "acute" and "chronic" rabbits in the [illegible] study responded to HCG treatment in a similar manner cannot easily be reconciled [illegible] this explanation.

[illegible] conclusion, by using a relatively novel approach we were able to demonstrate that an [illegible] in progesterone levels by increased ovarian output under physiological conditions [illegible] a pronounced and immediate depressing effect on the character of myometrial activity

[illegible] grateful to Professor Lars Philip Bengtsson for his advice and encouragement. We thank Miss [illegible] Gerle-Wickholm for her excellent technical assistance. This study was supported by the Ford [illegible], the Swedish Medical Research Council (project No 4781), Lund University and T & S [illegible] Stiftelse. T of the authors (T. L. and M. Å.) are grateful to AB Ferring, and Föreningen Liv [illegible] respectively for stipends.

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Acta physiol. scand. 1979 105. 420–427

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Effect of plasma H^+ ion concentration on pancreatic HCO_3^- secretion

By

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Abstract

RÆDER, M A MO and S AUNE. *Effect of plasma H -ion concentration on pancrea* *HCO_3 secretion* Acta physiol. scand 1979 *105* 420–427

The relationship between the rate of pancreatic HCO_3^- secretion and plasma H -ion concentration was in- vestigated in 15 pentothal anesthetized, secretin infused pigs (1.8 C.U./kg b.w h^{-1} intravenously) during acute metabolic and respiratory acid-base disturbances. Pancreatic HCO_3 secretion increased to 196±1… of control duri g alkalosis nd fell to 41±4% of control during acidosis. Partial metabolic compensa… of respiratory acidosis restored HCO^- secretion to 87±6% of control. A proportional relationship w… found between HCO^- secretion nd plasma pH Different, proportional relationships were found betwe… HCO secretion and plasma HCO concentration d ring metabolic and respiratory acid-base chang… HCO^- secretion was ndependent of H -ion concentration in pancreatic juice. Plasma H -ion concentra- tion, therefore, seems to determine the rate of pancreatic HCO secretion. This finding supports the hyp… thesis that proton pump is responsible for pancreatic HCO secretion.

Key words. Acid-base back-leakage hypothesis pigs, proton pump secretin

Some 90% of bicarbonate secreted by the pancreas derives from plasma bicarbonate (Ca… *et al* 1970) Correspondingly the rate of pancreatic bicarbonate secretion is sensitive t… changes in systemic acid-base balance. Previous studies on artificially perfused pancrea… have indicated that the rate of secretion of bicarbonate varies in proportion to the bicar- bonate content of the perfusate. (Case *et al* 1970 Schulz 1972) Other investigators, how- ever claim additive effects of perfusate pH and bicarbonate concentration on the secretor… rate (Swanson and Solomon 1975) Since bicarbonate secretion implies equimolar transpor… of H ions into interstitial fluid by secretory cells, a simple hypothesis would be that varia- tions in rate of pancreatic bicarbonate secretion during acid-base changes were due to varying back leakage of H -ions into secretory cells. If so extracellular H ion concentration would determine the rate of bicarbonate secretion from the pancreas. The first purpose of the present study therefore, was to examine what relationship pertains between plasma H -ion concentration and rate of pancreatic bicarbonate secretion in the intact animal.

The effects of varying plasma H -ion concentration on the rate of pancreatic bicarbonate secretion were examined at normal and high arterial P_{CO_2} in anesthetized pigs during laparotomy and continuous i.v. infusions of secretin. By increasing arterial P_{CO_2}, arterial pH may be lowered while maintaining arterial bicarbonate concentration. If arterial H -ion concentration determines the rate of pancreatic bicarbonate secretion, pancreatic bicarbonate output would parallel arterial pH. Alternatively, were arterial bicarbonate concentration determinant of the rate of pancreatic bicarbonate secretion, bicarbonate output would vary reciprocally with arterial bicarbonate concentration at normal and high arterial

The second purpose of the present study was to examine the effects on bicarbonate secretion of increasing H -ion concentration in pancreatic juice. At high P_{CO_2}, CO permeates the pancreatic duct epithelium, hydrates in the lumen and raises H -ion concentration in pancreatic juice. If as maintained by some authorities, pancreatic bicarbonate secretion is a consequence of active H -ion transport across the luminal cell membrane into bicarbonate secreting cells (Schulz et al. 1971, Schulz 1972, Simon and Kather 1975, Simon and Kather 1976, Wizemann and Schulz 1973), the raising of luminal H -ion concentration would be expected to enhance the rate of bicarbonate secretion due to reduced concentration differences against which the H -ions have to be transported. Alternatively, if active H -ion transport were confined to the interstitial cell membrane of bicarbonate secreting cells, alterations in luminal H -ion concentration would be expected to have no effect on H -ion transport rate, because the luminal H -ion concentration changes would be fully segregated from the active transport site.

We found that during constant i.v. secretin infusion, arterial pH determined the rate of pancreatic bicarbonate secretion at normal as well as high arterial P_{CO_2}. Different, proportional relationships were found between the rate of bicarbonate secretion and plasma bicarbonate concentration at normal and high arterial P_{CO_2}. Pancreatic juice H -ion concentration more than doubled during high P_{CO_2}. At comparable plasma pH, however, there was no difference in rates of pancreatic bicarbonate secretion during normal and high P_{CO_2}. These findings are compatible with the hypothesis that pancreatic bicarbonate secretion is a consequence of active transport of H -ions across the interstitial cell membrane of bicarbonate secreting cells in the pancreas.

Methods

Experiments were carried out on young pigs, weighing 20-25 kg, under general pentothal anesthesia (... i.v.) and supplementary doses were given ... as required, during the expts. An endotracheal tube was inserted ... The animals were artificially ventilated by breathing air/oxygen mixture at 4 ... CO_2 being added to the gas mixture when desired, to increase arterial P_{CO_2}. Anesthesia was maintained by i.v. ... administration of pancuronium bromide (Pavulon).
The carotid was cannulated for arterial blood sampling and blood pressure monitoring by means of a ... transducer (P23Gb). The jugular vein was cannulated for infusions. The abdomen was opened by ... midline incision. The main pancreatic duct was cannulated by ... soft, then polyethylene catheter ... duodenotomy for timed collection of pancreatic secretion. The gallbladder and urinary bladder were ... drained by indwelling Foley catheters. The common bile duct was ligated immediately ... the duodenum, and the pylorus was closed by ... stout silk ligature. The vagal innervation of the ... was left intact. Following insertion of catheters, the abdominal incision was closed by towel clips.

An electric pillow placed under the animal, ensured constant body temperature during the expts. 100-1[illegible] ml of 0.9 NaCl was infused i.v. during the surgical procedure to expand extracellular fluid volume [illegible] maintain good peripheral circulation. Continuous stimulation of pancreatic secretion was ensured [illegible] i.v. infusion of secretin (from the Karolinska Institute, Stockholm, Sweden) at 1.8 C.U./kg h. [illegible] gave a maximal secretion of HCO_3 in the pig.

During steady state two 4 min collections of pancreatic juice were made. These samples are referred as *control*. Arterial blood specimens were drawn in the middle of each control period for electrolyte [illegible] blood gas analysis. Following timed collection of pancreatic juice, 300 μl of pancreatic juice was collected anaerobically for immediate H -ion and P_{CO_2} determination.

Respiratory acidosis (hypercapnia) was induced by adding CO to the inspired air/oxygen [illegible] aiming at an arterial P_{CO_2} of 100–150 mmHg. During steady state, timed samples of pancreatic juice [illegible] blood specimens were obtained as above for analysis (14 animals).

Compensated respiratory acidosis was obtained through i.v. infusion of isotonic $NaHCO_3$ ([illegible] mmol/over 30 min) during sustained elevation of arterial P_{CO_2}. Several pancreatic juice and blood [illegible] were obtained as above during back titration of arterial pH with $NaHCO_3$ (5 animals).

Metabolic acidosis was induced by i.v. infusion of 100–150 mmol HCl, dissolved in 1 000 ml H_2O, [illegible] 30 min. Pancreatic juice and blood specimens were obtained as above. Normal acid-base balance was [illegible] sequently restored by infusion of 4.2 $NaHCO_3$, with frequent monitoring of arterial pH and blood [illegible] parameters. A new set of pancreatic juice and blood samples was obtained upon regaining normal acid-base balance and included in the control group (8 animals).

Metabolic alkalosi was obtained through i.v. infusion of 250–330 mmol isotonic $NaHCO_3$ over [illegible] min. Blood specimens and pancreatic juice samples were obtained as above (6 animals). All animals [illegible] killed at the end of the expts.

Pancreatic juice and plasma H -ion concentrations and P_{CO_2} were determined by an Instrumentation Laboratories Blood Gas Analyzer 313 (Lexington, Mass., U.S.A.). Plasma HCO concentration was calculated according to Henderson Hasselbalch's formula HCO_3 concentration = antilog (pH [illegible]) [illegible] P_{CO_2}. Pancreatic juice HCO concentration was estimated as the difference between the sum of Na [illegible] K concentrations and Cl^- concentration in pancreatic juice. Plasma and pancreatic juice Na and [illegible] concentrations were measured by an Instrumentation Laboratories 343 flame photometer. Chloride determinations were made with a CM 10 chloride titrator (Radiometer, Copenhagen, Denmark).

Statistical methods

Wilcoxon's signed rank test for paired samples and Wilcoxon's rank test for unpaired measurements (Mann-Whitney test) were used to evaluate differences between observations (Snedecor 1967). Linear regression were calculated using the method of least squares fit. Multiple regression was calculated using independent variables (Snedecor 1967).

Results

Changes in systemic acid base balance had pronounced effects on pancreatic bicarbonate secretion (Table I). During respiratory acidosis, pancreatic bicarbonate secretion fell by [illegible] average of 66 ± 6% during metabolic acidosis, pancreatic bicarbonate secretion fell by [illegible] average of 49 ± 6%. Metabolic alkalosis greatly increased pancreatic bicarbonate secretion. On average, secretion doubled during NaHCO infusion at normal P_{CO_2}. At high P_{CO_2} NaHCO infusion restored plasma H ion concentration and pancreatic bicarbonate secretion towards control.

Effect of plasma H ion concentration on pancreatic HCO secretion

The rate of pancreatic bicarbonate secretion increased with plasma pH during normal [illegible] high P_{CO_2}. Fig. 1 indicates a linear relationship between the rate of secretion and plasma pH the correlation coefficient being 0.82. Pancreatic bicarbonate secretion was measured down to pH 6.95—secretion could be halted by allowing plasma pH to fall below this value

	Arterial concentrations			Pancreatic juice concentrations						Pancreatic secretion			
	H nmol/l	HCO_3 mmol/l	P_{CO_2} mmHg	I mmol/l	HCO_3 mmol/l	P_{CO_2} mmHg	Na mmol/l	K mmol/l	Cl^- mmol/l	Volume ml/min	CO^- µmol/min	HCO_3	
Control	38 ± 1	26.4 ± 0.6	41 ± 1	6 ± 0	147 ± 1	33 ± 1	156 ± 1	4.9 ± 0.1	14 ± 1	1.18 ± 0.07	174 ± 11	100 ± 2	−31
Respiratory acidosis	101 ± 5	33.7 ± 2.3	138 ± 5	27 ± 2	135 ± 4	130 ± 7	159 ± 2	4.7 ± 0.4	24 ± 3	0.45 ± 0.01	65 ± 19	34 ± 6	−12
Metabolic acidosis	69 ± 5	14.8 ± 1.0	43 ± 2	10 ± 3	131 ± 3	45 ± 3	146 ± 1	5.6 ± 0.2	21 ± 2	0.66 ± 0.08	89 ± 12	51 ± 6	−11
Metabolic alkalosis	26 ± 2	39.3 ± 2.3	41 ± 2	6 ± 0	160 ± 2	37 ± 2	162 ± 2	4.3 ± 0.2	7 ± 1	2.40 ± 0.13	385 ± 22	196 ± 10	−12
Compensated respiratory acidosis	74 ± 4	49.9 ± 3.5	151 ± 4	21 ± 1	161 ± 3	114 ± 12	166 ± 2	4.1 ± 0.2	10 ± 2	1.14 ± 0.12	186 ± 22	87 ± 6	−5

Data presented are mean values ± S.E. from 15 expts. on anesthetized young pigs (20–25 kg b.w.). Control normal plasma H -ion concentration and normal P_{CO_2}. Respiratory acidosis – addition of CO_2 to inspired air/O_2 mixture. Metabolic acidosis – following infusion of 100–150 mmol HCl l over 30–40 min. Metabolic alkalosis – following infusion of 1.5–2 liters 1.2 % $NaHCO_3$ l over 30–40 min. Compensated respiratory acidosis – following infusion of 1.0–1.5 liters 1.2 % $NaHCO_3$ over 30–40 min. ~ per cent of value during control, n number of analyzed pancreatic juice samples.

An electric pillow placed under the animal, ensured constant body temperature during the expts. [illegible] ml of 0.9 NaCl was infused i.v. during the surgical procedure to expand extracellular fluid volume [illegible] maintain good peripheral circulation. Continuous stimulation of pancreatic secretion [illegible] i.v. infusion of secretin (from the Karolinska Institute, Stockholm, Sweden) at 1 8 C.U./kg [illegible] gave a maximal secretion of HCO in the pig.

During steady state two 4 ml collections of pancreatic juice were made. These samples [illegible] as *control*. Arterial blood specimens were drawn in the middle of each control period for electrolyte [illegible] blood gas analysis. Following timed collection of pancreatic juice 300 μl of pancreatic juice was [illegible] anaerobically for immediate H^+-ion and P_{CO_2} determination.

Respiratory acidosis (hypercapnia) was induced by adding CO_2 to the inspired air [illegible] aiming at an arterial P_{CO_2} of 100–150 mmHg. During steady state, timed samples of pancreatic juice [illegible] blood specimens were obtained as above for analysis (14 animals).

Compensated respiratory acidosis was obtained through i.v. infusion of isotonic $NaHCO_3$ [illegible] mmol/over 30 min) during sustained elevation of arterial P_{CO_2}. Several pancreatic juice and blood [illegible] were obtained as above, during back titration of arterial pH with $NaHCO_3$ (5 animals).

Metabolic acidosis was induced by i.v. infusion of 100–150 mmol HCl, dissolved in 1 000 ml H_2O [illegible] 30 min. Pancreatic juice and blood specimens were obtained as above. Normal acid-base balance was [illegible] sequently restored by infusion of 4 [illegible] $NaHCO_3$, with frequent monitoring of arterial pH and blood [illegible] parameters. A new set of pancreatic juice and blood samples was obtained upon regaining normal acid [illegible] balance and included in the control group (8 animals).

Metabolic alkalosis was obtained through i.v. infusion of 250–330 mmol isotonic $NaHCO_3$ over [illegible] min. Blood specimens and pancreatic juice samples were obtained as above (6 animals). All animals [illegible] killed at the end of the expts.

Pancreatic juice and plasma H^+-ion concentrations and P_{CO_2} were determined by an Instrumentation Laboratories Blood Gas Analyzer 313 (Lexington, Mass., U.S.A.). Plasma HCO_3^- concentration was [illegible] culated according to Henderson-Hasselbalch's formula HCO_3 concentration = antilog (pH 6.1) [illegible] P_{CO_2}. Pancreatic juice HCO concentration was estimated as the difference between the sum of Na [illegible] K concentrations and Cl^- concentration in pancreatic juice. Plasma and pancreatic juice Na [illegible] concentrations were measured by an Instrumentation Laboratories 343 flame photometer. Chloride de[illegible] minations were made with a CM 10 chloride titrator (Radiometer, Copenhagen, Denmark).

Statistical methods

Wilcoxon signed rank test for paired samples and Wilcoxon's rank test for unpaired measurements (Mann-Whitney test) were used to evaluate differences between observations (Snedecor 1967). Linear regressions were calculated using the method of least squares fit. Multiple regression was calculated using independent variables (Snedecor 1967).

Results

Changes in systemic acid base balance had pronounced effects on pancreatic bicarbonate secretion (Table I). During respiratory acidosis, pancreatic bicarbonate secretion fell by [illegible] average of 66 ± 6% during metabolic acidosis, pancreatic bicarbonate secretion fell by [illegible] average of 49 ± 6%. Metabolic alkalosis greatly increased pancreatic bicarbonate secretion. On average, secretion doubled during NaHCO infusion at normal P_{CO_2}. At high P_{CO_2} NaHCO infusion restored plasma H^+-ion concentration and pancreatic bicarbonate secretion towards control.

Effect of plasma H^+-ion concentration on pancreatic HCO_3^- secretion

The rate of pancreatic bicarbonate secretion increased with plasma pH during normal [illegible] high P_{CO_2}. Fig. 1 indicates a linear relationship between the rate of secretion and plasma pH, the correlation coefficient being 0.82. Pancreatic bicarbonate secretion was measured down to pH 6.95—secretion could be halted by allowing plasma pH to fall below this val[illegible]

-lower the rate of pancreatic bicarbonate secretion was significantly lower at any -ma HCO_3 concentration during hypercapnia than during normocapnia. Thus, at plasma -O_3 concentration of 25 mmol/l, pancreatic bicarbonate secretion averaged 103% during -ocapnia and 16% during hypercapnia.

-ect of plasma P_{CO_2} on pancreatic juice composition

-percapnia increased pancreatic juice P_{CO_2} and H -ion concentration (Table I). During -rmocapnia, the juice H -ion concentration ranged between 5–10 nmol/l and remained -ntially independent of plasma H -ion concentration. Hypercapnia increased juice -ion concentration to 23 ± 1 nmol/l ($p < 0.05$). The increase in the juice H -ion concentra-n was paralleled by an increase in P_{CO_2} in pancreatic juice from 41 ± 1 mmHg to 149 ± 3 -mHg. However despite this increase P_{CO_2} in pancreatic juice was still 27 ± 4 mmHg below -terial P_{CO_2}.

There was no consistent relationship between the rate of pancreatic bicarbonate secretion -d pancreatic juice P_{CO_2}. Similarly the rate of bicarbonate secretion exhibited no con--tent relationship to H -ion concentration in pancreatic juice.

Discussion

-e present study demonstrates that plasma H -ion concentration determines the rate of -creatic bicarbonate secretion in the intact animal. Previous studies on isolated organ -parations have indicated that the secretion rate is proportional to the perfusate HCO -centration (Case *et al* 1970 Schulz 1972) and that the perfusate pH and the HCO_3 -centration have additive effects on the secretion rate (Swanson and Solomon 1975). -wever by relating the rate of bicarbonate secretion to plasma pH and HCO concentra- -s during normocapnia and hypercapnia, it was possible, in the present study to dissociate -e effects of plasma H -ion and plasma HCO_3 concentration on the bicarbonate secretory -chanism. We found that alterations in the secretion rate during variations in plasma -CO concentration could be accounted for by the concomitant changes in plasma pH. -erefore, at normal and high P_{CO_2}, plasma pH and hence interstitial fluid pH, determines -e rate of pancreatic bicarbonate secretion.

Asymmetry of sensitivity of the bicarbonate secretory mechanism to extracellular H -as was demonstrated, there was no definite effect on bicarbonate transport of increasing -e luminal H -ion concentration in pancreatic ducts, as during high P_{CO_2}, while changes in -e H -ion concentration on the interstitial side of the secretory cells elicited large changes -the rate of bicarbonate secretion. Since bicarbonate secretion implies equimolar transport -f H -ions across the interstitial cell membrane of bicarbonate secreting cells, a simple ex--lanation of the asymmetric sensitivity of the secretory mechanism to extracellular H -ns -ould be that interstitial H -ions interfere with the activity of a proton pump located -ose to or in the interstitial cell membrane.

There are several w-ys in which interstitial H -ions might impair the activity of a proton -ump. One hypothesis would be that interstitial H -ions regulate the net transport rate by -aking back through the interstitial cell membrane into bicarbonate secreting cells. The

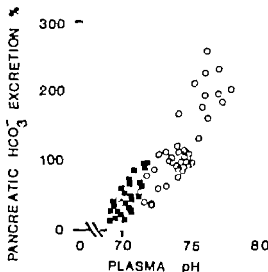


Fig. 1

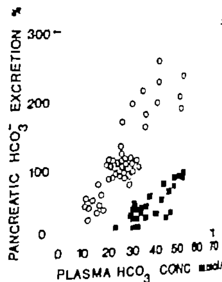


Fig. 2

Fig. 1 Relationship between rate of pancreatic HCO^- secretion and plasma pH during acute changes systemic acid-base balance. ○ arterial P_{CO_2} = 42±1 mmHg. ■ arterial P_{CO_2} = 145±3 mmHg HCO secretion is calculated as per cent of control. Fo definitions of acid-base disturbances, see footnote Table Secretin was infused f t 1.8 C.U./kg b.wt. h N mber of expts. = 15

Fig. 2. Relationship between pancreatic HCO secretion and plasma HCO_3^- concentration during changes in systemic acid-base balance. Symbols and definitions as in Fig. 1 Number of expts 15

through administration of high P_{CO_2} in the respiratory gas mixture. This effect of plasma on secretion was reversible, as is evident by the return of secretion to control values up restoration of normal plasma pH whether this was achieved through normalization plasma P_{CO_2} or through intravenous infusion of NaHCO (Table I).

Multiple regression analysis of the effect of plasma pH and P_{CO_2} on the rate of pancre bicarbonate secretion at plasma pH < control yielded the following equation.

Rate of HCO secretion = 1 074 54 + 157 49 pH + 0 054 P_{CO_2}. S.E. = ±15 1

If P_{CO_2} is omitted the equation becomes

Rate of HCO secretion = −1 006.75 + 148 64 pH S E. = ±15.2

Thus, the standard error of a multiple regression analysis, including plasma P_{CO_2} is nearly the same as the standard error of a simple regression calculated for plasma pH rate of pancreatic bicarbonate secretion This indicates that plasma P_{CO_2} is not an import determinant of pancreatic bicarbonate secretion under the conditions of normal and su normal plasma P_{CO_2} and H + ion concentrations.

Relationships between plasma HCO concentration and pancreatic bicarbonate

Pancreatic bicarbonate secretion increased in proportion with plasma bicarbonate con tration at normal and high P_{CO_2} (Fig. 2) the correlation coefficients being 0.84 and 0 respectively

owever, the rate of pancreatic bicarbonate secretion was significantly lower at any ma HCO_3 concentration during hypercapnia than during normocapnia. Thus, at plasma O_3^- concentration of 25 mmol/l, pancreatic bicarbonate secretion averaged 103% during ocapnia and 16 „ during hypercapnia.

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Asymmetry of sensitivity of the bicarbonate secretory mechanism to extracellular H s demonstrated there was no definite effect on bicarbonate transport of increasing luminal H -ion concentration in pancreatic ducts, as during high P_{CO_2} while changes in e H -ion concentration on the interstitial side of the secretory cells elicited large changes the rate of bicarbonate secretion. Since bicarbonate secretion implies equimolar transport H -ions across the interstitial cell membrane of bicarbonate secreting cells, a simple ex lanation of the asymmetric sensitivity of the secretory mechanism to extracellular H s ould be that interstitial H -ions interfere with the activity of proton pump located ose to or in the interstitial cell membrane.

There are several ways in which interstitial H -ions might impair the activity of a proton ump. One hypothesis would be that interstitial H -ions regulate the net transport rate by aking back through the interstitial cell membrane into bicarbonate secreting cells. The

simplest concept would be that back leakage of H^+ ions would occur through leak pat ways of fixed proton conductance. Accordingly the rate of back-leakage would be e pected to be proportional to plasma H -ion concentration. The results of the present i vestigation indicate a more complex relationship between the plasma H -ion concentratio and the rate of back leakage, and therefore argue against such a simplified model. Alt natively H^+ ions may leak back through the cell membrane by combining with a membran bound carrier Such a carrier mediated diffusion would be expected to approach a transpo maximum as interstitial H ion concentration increases. The observed plasma pH effect secretion in the present study may be interpreted within this framework. Assuming th there is a constant rate of H ion secretion during constant secretin infusion, the obser diminution in rate of bicarbonate secretion and hence net H -ion transport rate, could due to carrier mediated back-leakage of H ions leakage would be proportional to plas H ion concentration during alkalosis and would become progressively less dependent plasma H -ion concentration at normal plasma pH and during acidosis, due to mounti carrier saturation with H -ions.

The possibility remains that external H ions may interfere with proton pump acti through an inhibitory action on oxidative metabolism coupled to active H -ion transpo This effect of external H ions on proton pumps has been claimed to exist in other tiss (Beauwens and Al-Awqati 1976) There are no available data on pancreatic metabolic ra during acid base disturbances, to either refute or validate such a hypothesis.

It is of interest to note how the findings of the present study otherwise parallel the featu of the proton pump in turtle bladder epithelium. Turtle bladder epithelium possesse similar asymmetry in sensitivity to external H -ions an increase in H -ion concentrati on the serosal side of the epithelium has no effect on H -ion secretion (Beauwens and A Awqati 1976), while an increase in H ion concentration on the mucosal side, which believed to be the aspect of the cell where the proton pump is located, depresses the rate H ion secretion (Beauwens and Al Awqati 1976, Schwartz *et al* 1972, Steinmetz 1967)

Currently there are two main hypotheses on the cellular mechanism of pancreatic carbonate secretion The proton pump hypothesis, advanced by Davies (1949), propo that H ions are secreted into blood from pancreatic cells concurrently HCO_3 ions p into the secretory ducts

The alternative HCO_3-ATPase hypothesis is based on the finding of HCO_3-ATPase membrane fragments of pancreatic tissue (Simon *et al* 1972, Simon and Thomas 19 Wizemann and Schulz 1973 Wizemann *et al.* 1974) HCO_3-ATPase has been suggested function either as an anion transport mechanism (Schulz *et al.* 1969 Simon *et al* 1972), as a proton pump in the luminal cell membrane (Schulz *et al* 1971 Schulz 1972, Simon a Kather 1976 Wizemann and Schulz 1973) A proton pump in this location would be versed in the sense that it would actively transport H -ions from the pancreatic duct lum into ductal epithelial cells. If responsible, HCO_3-ATPases would exhibit maximal acti during stimulation under physiological conditions, since *in vitro* studies indicate that maxim enzyme activity is reached at ambient HCO concentrations of 25 μmol/l (Simon and Th mas 1972). Furthermore, pH sensitivity of this enzyme is not very marked (Simon a Thomas 1972). Therefore, any effects of plasma H ions on such an HCO_3-ATPase mec

... ion could necessarily be due to H -ion leakage into pancreatic juice, with varying short ...coming of the HCO_3-ATPase pump.

The findings of the present study argue against this possibility: during high P_{CO_2} pancreatic juice H -ion concentration increased significantly thus reducing the chemical driving force for H -ion back-leakage. However changes in P_{CO_2} per se did not significantly change the rate of pancreatic bicarbonate secretion in our experiments.

In conclusion, it appears that plasma H -ion concentration determines the rate of pancreatic bicarbonate secretion in the intact animal. H -ion concentration in pancreatic juice, however, does not appear to influence bicarbonate output. Asymmetry in effects of environmental H -ions is compatible with the hypothesis of a proton pump in the interstitial cell membrane of bicarbonate secreting cells.

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Pregnancy induced alterations in the turnover rate of ^{3}H noradrenaline formed from ^{3}H tyrosine in guinea-pig uterus

By

G Thorbert P Alm Ch. Owman and N-O Sjöberg

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Abstract

Thorbert G P Alm Ch Owman and N-O Sjöberg *Pregnancy induced alterations in the turnover rate of ^{3}H-noradrenaline formed from ^{3}H-tyrosine in guinea-pig uterus.* Acta physiol scand. 1979 *105* 428–436.

The rate of noradrenaline (NA) turnover in the non-pregnant and pregnant guinea-pig uterus was determined after the administration of the labelled precursor tyrosine. Endogenous NA was determined fluorimetrically. In non-pregnant animals the turnover of ^{3}H NA was slower in the uterine horn ($t_{1/2}$ ...) and cervix ($t_{1/2}$ = 9 h) than in the reference organ, heart ($t_{1/2}$ = 6 h). During pregnancy (60–65 days *post coitum*) endogenous NA in the heart increased in pace with tissue weight gain, yet, in the uterus there was a drastic reduction, down to zero levels. ^{3}H NA turnover increased significantly in the cervix, no ^{3}H-NA formation remained in the uterine horn (a situation resembling that following 6-hydroxydopamine sympathectomy of non-pregnant animals), and the turnover was unaltered in the heart. The observations confirm the general idea illustrated by the results in the heart, of a functional adaptation with a constant neuron-target relationship during increased physiological demands. They also confirm that a very peculiar and complex neuron-target relationship develops in the uterus during pregnancy—involving functional and structural denervation of the adrenergic nerve plexus in the uterine horn, whereas an increased activation might explain the changes in neuronal NA and its turnover in the cervix.

The adrenergic innervation in the uterus of guinea pig and many other species including man undergoes dramatic changes during pregnancy associated near term with a reduction in the level of the noradrenaline (NA) content to near zero values in both the uterine horn and the cervix (Owman *et al* 1975) In the uterine tissue surrounding the fetus (perifetal uterus), the *in vitro* activity of tyrosine hydroxylase (Alm *et al* 1979 a) and the axonal uptake of ^{3}H NA (Alm *et al* 1979 b) are lowered to non-detectable levels. These changes seem to reflect a widespread and total peripheral adrenergic degeneration of the adrenergic nerve plexus in the perifetal uterus (Thorbert *et al* 1978 b Sporrong *et al* 1979) In the cervix no alterations have been observed in these two parameters (Alm *et al.* 1979 a, Alm *et al.* 1979 b). A more or less intact nerve plexus was found in this region by the use of α-methyl-NA incubations (Thorbert *et al* 1978 b) There is at present no explanation for the cause of the transmitter decay in the cervix (Owman *et al* 1975)

In order to relate the above mentioned changes to a more dynamic aspect of adrenergic [illegible], the formation of ^{3}H NA from H-tyrosine was studied in the guinea-pig uterus before and during pregnancy and compared with that following chemical sympathectomy with 6-hydroxydopamine (6-OH-DA).

Materials and Methods

Animals. Female guinea-pigs of [illegible] mixed strain were used. Sexually mature virgin animals (400–500 g b.wt.) in the [illegible] stage, as judged from the closed vagina, were selected. Chemical sympathectomy [illegible] as performed [illegible] virgin animals by [illegible] injections of 6-hydroxydopamine with one day interval between each [illegible] (100 mg/kg dissolved in 0.9 % saline containing 0.2 mg/ml of ascorbic acid). Animals at 60–65 days of pregnancy were obtained by palpation. After killing under light ether anaesthesia the day of pregnancy was determined by measurement of mean weight and crown-rump length of the fetuses (Draper 1920, [illegible] 1969).

Experimental procedure. A total of 55 animals received 5 μg/kg ^{3}H-tyrosine (—ring—3,5—^{3}H 51 Ci/[illegible], New England Nuclear) into the jugular vein during halothane anaesthesia (Halothan, Hoechst AG). At various time intervals they [illegible]ere sacrificed and the heart and [illegible]hole uterus were rapidly dissected out. [illegible] latter organ was divided into the cervix and uterine horns (only one used for analysis). I[illegible] the case of [illegible] pregnancy (4 animals) both uterine horns were analyzed separately. The tissues [illegible]ere dried between blotting papers, weighed, and homogenized in ice-cold 0.4 N perchloric acid. Aliquots of the tissue [illegible] were further processed essentially according to Persson and Waldeck (1968 see also Persson 1970) [illegible] separate the ^{3}H-catechol compounds formed and their corresponding ^{3}H-methoxylated metabolites on [illegible] columns. Petroleum hydroxide was used instead of potassium carbonate for pH adjustment of the [illegible] extracts. T[illegible] get stable recoveries, 50 μg of unlabelled noradrenaline, dopamine, normetanephrine [illegible] methoxytyramine were added together to the respective aliquot of homogenate. The ^{3}H-catechol fraction obtained were applied on ion exchange columns (Dowex 50 W X4, diameter 4 mm, length 140 mm [illegible] pH 1) to separate the ^{3}H-NA and ^{3}H-DA formed by the differential elution with 1N and 2N HCl (Persson and Waldeck 1968, Persson 1970). The hydrochloric acid eluates of ^{3}H-NA and [illegible]H-DA were put in [illegible] vials and evaporated to dryness. The radioactive residues were taken up in 1 ml ethanol containing 0.1 [illegible] HCl, and 9 ml scintillation solution was added (5 g PPO = 2.5 diphenyloxazole, and 0.3 g [illegible] POPOP 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene per liter toluene). Radioactivity was [illegible] in [illegible] liquid scintillation spectrometer and expressed as dpm per g tissue (specific activity) or as [illegible] per organ (total activity). Quenching [illegible]as corrected for using external standardization. Recovery for NA was [illegible] 1.51 (n [illegible] 18) and for DA 75.57 ± 1.41 % (= 18). No corrections for recovery were made. No detectable ^{3}H-DA was found in any of the different organs investigated.

Endogenous NA in cardiac and uterine tissues was extracted by perchloric acid, separated on cation exchange (Dowex) columns and finally the amount present determined fluorimetrically (Bertler *et al.* [illegible] 1961).

Statistics. In text and figures, multiple observations are given as arithmetical mean ± S.E., [illegible] = number of observations. Observed differences in means [illegible]ere evaluated using Students' *t*-test. In the analyses of the disappearance of ^{3}H-NA after ^{3}H-tyrosine injection the values for dpm/mg were expressed as $\log_{10}$. Regression lines were calculated according to the method of least squares. Conventional statistical methods were used for tests of linearity and slope. Differences in slopes were evaluated according to Pugsley (1946). The rate constant (k) for the decline in ^{3}H-NA [illegible]as calculated according to the relationship k [illegible] slope [illegible] ln 10. Apparent half lives of ^{3}H-NA [illegible]as calculated as ln 0.5/k (Goldstein, Aronow and Kalman 1969).

Results

The level of endogenous NA in the various tissues and the amount of ^{3}H-NA formed under the various experimental conditions are summarized in Table I.

Heart. Total content of endogenous NA was increased by 66 % in pregnant animals compared to virgin controls ($p < 0.001$). This increase was parallel to the weight gain observed in pregnancy and, accordingly no significant difference was seen in the NA con-

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[...]E I. Endogenous NA and ^{3}H-NA formed from ^{3}H-tyrosine in uterus and heart under various ex[...]perimental and reproductive conditions. Values are means ± S.E. n = number of determinations.

[...]gan	Reproductive state and experimental condition	NA content (µg/organ)	NA concentration (µg/g)	n	^{3}H-NA formed per organ at 4 h (dpm)	^{3}H NA formed per g tissue at 4 h (dpm)	n
[...]art	Non-pregnant	2 47±0.16	2.07±0 13	6	12 863±2 290	10 384±1 969	4
	6-OH DA	0 13±0 02	0 08±0.01	5	1 310± 96	1 076± 141	4
	Pregnant	4 10±0.25	1.81±0.14	5	35 022±2 670	13 182±1 002	4
[...]rvix	Non-pregnant	0 28±0 02	1 40±0 07	6	902± 251	2 390± 443	4
	6-OH-DA	0 03±0 00	0 09±0.01	5	36± 21	196± 113	4
	Pregnant	0 02±0.01	0 03±0 01	5	1 879± 249	881± 162	4
[...]rine [...]rn	Non-pregnant	0 31±0 02	1.23±0 11	6	813± 160	2 081± 375	4
	6-OH DA	0 02±0 00	0 06±0 01	5	24± 24	128± 128	4
	Pregnant	0 01±0 00	0 00±0.00	5	non-detectable	non-detectable	4

[...]t and non-pregnant animals (Table II). In agreement with this, the apparent half-lives [...] the turnover were very similar in the hearts of the two groups 5.7 h in non-pregnant and [...] h in pregnant animals. The turnover rate in the heart of non-pregnant animals was [...]ter than in the cervix (p 0.10) and uterine horn ($p < 0.05$)

Cervix. At term pregnancy the total amount of endogenous NA was reduced to 7% com[...]red to non-pregnant animals. Due to weight gain of the organ during pregnancy the [...]duction in tissue concentration of endogenous NA was even more pronounced, being [...]y 2 of controls. At term pregnancy the total amount of ^{3}H NA formed had clearly [...]reased (p 0 01), whereas the specific activity of H NA was reduced to one third (p < [...]) compared to non-pregnant animals. Parallel reduction in endogenous NA and in [...]e ^{3}H-NA formed was seen after 6-OH DA injection.

The highest figures for H-NA formed from ^{3}H-tyrosine were seen at 4 h in term preg[...]cy and at 1 h in the non-pregnant animals. From these peak values the tissue ^{3}H-NA [...]creased. The reduction in log total tissue ^{3}H NA activity was linear with regard to time [...]th in pregnant (p 0 025) and non-pregnant (p 0 05) animals. The negative slope was [...]nificantly different from zero in pregnant ($p < 0.001$) as well as non-pregnant animals [...] 0 005). However the magnitude of this negative slope was almost 3 times greater in the [...]egnant animals (Table II) and, accordingly the apparent half-lives for the turnover was [...]o shorter about 4 h. This difference in slope was significant ($p < 0.01$).

[...]LE II. Values for slope (k) and apparent half-life ($t_{1/2}$) of transmitter turnover based on regression analyses of changes in tissue H-NA after ^{3}H-tyrosine injection. Statistical evaluation of differences in k-values in non-pregnant (NP) and pregnant (P) animals, n.s. = non significant.

[...]gan	Reproductive state	k ± S D.	$t_{1/2}$ (h)	$k_{NP} + k_P$
[...]rt	Non-pregnant	0.121±0 024	5.7	
	Pregnant	0.117_0 043	5 9	n.s.
[...]rvix	Non-pregnant	0 077_0 020	9 1	
	Pregnant	−0.185_0 033	3 7	p 0 01
[...]rine horn	Non-pregnant	0 067_0 020	10 4	
	Pregnant	0	—	—

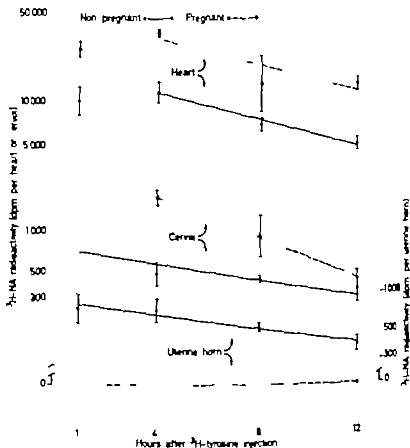


Fig. 1 The level of ³H NA formed and the rate of its turnover in heart and uterus following a single injection of ³H tyrosine Maximum ³H NA was seen within 1 h in the non-pregnant uterus, and at 4 h in the other tissues. The log ³H NA decay was linear with time. No ³H NA formation occurred in pregnant uterine horn Number of determinations at each point = 4. The values for slope and half-life together with statistical analysis, are presented in Table II

centration of the heart The amount of ³H NA formed from ³H-tyrosine at 4 h after injection varied in a similar way Thus, ³H NA was considerably higher in the hearts of pregnant animals when calculated per whole organ but no significant difference was found if ³H NA was expressed in terms of specific activity (dpm/g tissue w w) Chemical sympathectomy produced marked decreases in both total content (95%) and concentration (96%) of endogenous NA Also the ³H NA formation was drastically reduced by this drug only about 10% (irrespective if calculated per organ or on weight basis) ³H NA was formed compared to virgin control animals.

In hearts of both pregnant and non-pregnant animals the amount of ³H NA increased to 4 h, when the highest values were observed (Fig. 1) Subsequently 8 and 12 h after injection the amount of ³H NA formed decreased. There was a linear relationship (Fig. 1) between time and log radioactivity of ³H NA (dpm) formed per organ in the 4–12 h interval (non-pregnant, $p < 0.01$ pregnant, $p < 0.05$) and there was a significant negative slope of the two lines (non-pregnant $p < 0.001$ pregnant, $p < 0.025$) The slope (k) of the regression line, expressing the turnover rate of the ³H NA was virtually the same in the hearts of pr

present study indicate that significant amounts of ³H-tyrosine have been available for NA synthesis also in the interval between 1 and 4 h. The turnover and half-life estimates are based on the assumption that labelled precursor is no longer available during the period of ³H-NA decay (Persson 1969, Weiner 1974). Therefore the estimation of the turnover was started with the highest values for ³H NA formed. The figures obtained for turnover rate and apparent half-life in the heart of both pregnant and non-pregnant animals are well within the range reported by others (*cf* Östman and Nybäck 1976, Kennedy and Marshall 1977).

During pregnancy the weight of the heart and its NA content was considerably increased. In view of the fact that the transmitter concentration per unit weight remained unchanged, it is probable that the phenomenon represents an increased functional demand on the heart in pregnancy. Also the ³H NA formation from ³H-tyrosine was found to keep pace with the organ weight gain. The observations may be looked upon as examples of the preservation of a constant relationship between the terminal nerve plexus and its target tissue under physiological circumstances (Hendry 1976).

The musculature of the internal male genital organs is innervated exclusively by the special type "short adrenergic neurons" (Sjöstrand 1965, Owman, Sjöberg and Sjöstrand 1971, Baumgarten, Owman and Sjöberg 1975). This arrangement has made it possible to separately analyze pharmacological and physiological differences between these neurons and the classical sympathetic nerves, which arise in para- and prevertebral ganglia. Using a number of approaches it has been found that the short adrenergic neurons constitute a functionally unique system of nerves with several peculiarities recently listed and discussed by Baumgarten *et al* (1975). One is a particularly slow transmitter turnover (Swedin 1971). The short adrenergic neurons represent a considerable contribution to the sympathetic innervation of the guinea-pig uterus (Thorbert *et al.* 1977). From the results of fluorescence microscopy and fluorimetric determinations of NA after various types of pharmacological interference it has been suggested that the uterine adrenergic nerves of the guinea-pig have a low functional activity compared to the heart, possibly reflecting this marked contribution of short adrenergic neurons (Thorbert *et al.* 1978 b). The mixed population of adrenergic nerves is well reflected in the smaller difference in comparison with the heart than the previously reported difference between heart and vas deferens (Swedin 1971, Bralet, Beley and Bralet 1971). The direct measurement of ³H NA turnover in the present study confirmed these observations by showing a lower turnover rate in the uterine horns and cervix than in the heart. Also the oviduct of the rabbit has a mixed population of "long" and "short" adrenergic neurons (Owman, Rosengren and Sjöberg 1966). Based on the rate of disappearance of endogenous NA following synthesis inhibition with α-methyl-p-tyrosine, it was recently indicated that the NA turnover is higher in the heart than in the oviducts, supporting the concept that the adrenergic innervation of the genital tract represents a functionally different system of peripheral sympathetic nerves (Kennedy and Marshall 1977).

The adrenergic innervation in the uterine horns during the growth of the conceptus has been studied by various techniques in, particularly, the guinea-pig. A total disappearance of fluorescent adrenergic nerves (Thorbert *et al.* 1978 b) and a parallel drastic reduction in

Uterine horn Chemical sympathectomy by 6-OH DA reduced the content and concentration of endogenous NA by about 95% The total amount ³H NA formed from ³H-tyrosine was 3 and the corresponding specific activity of ³H NA 6 % of that in organs with intact adrenergic innervation Even more pronounced reductions were seen in late pregnancy where NA values were at or below the limit of detectability and no ³H NA formation could be established 4 of the animals injected with ³H-tyrosine (at 4 and 12 h) carried fetuses only in one of the uterine horns. Thus, the formation of H NA in the empty horn could be analyzed separately The radioactivity was below the level of detection also in these horns

The highest value for H NA formed in the uterine horns from non-pregnant animals was found at 1 h, and a decrease was observed with longer observation times (Fig. 1) The relationship between log tissue H NA and time was linear ($p < 0.05$). The turnover rate was very similar to that found in the cervix of non pregnant animals (half-life about 10 h) but slower than that in the heart ($p < 0.05$)

Discussion

Various methods have been used to estimate the NA turnover in adrenergic nerves (for reviews, see Costa 1972, Weiner 1974). One very frequently used approach involves measurement of the decrease in endogenous NA after synthesis inhibition by α-methyl-p-tyrosine This has certain limitations, *e.g* incomplete inhibition of the tyrosine hydroxylase (Widerlöw 1977) or change in neuronal activity caused by the interfered synthesis (Weiner 1974, Widerlöw 1977) Another method measures the disappearance of labelled NA injected in tracer doses (Costa 1972) In a recent study on incubated myometrial tissue slices we have shown that H NA is efficiently taken up from the incubation medium, and that the uptake can be separated into 3 shares neuronal uptake, extraneuronal uptake, and passive distribution in the extracellular space (Alm *et al* 1979 b) In that study it was shown that the neuronal uptake decreased drastically during pregnancy and that the extraneuronal accumulation was reduced to a smaller extent implying that this part of the total uptake becomes relatively more important during pregnancy For this reason, the use of H NA administration to estimate the rate of NA turnover in the pregnant myometrium has the obvious risk of determining predominantly the extraneuronal turnover of NA

In this particular situation, the measurement of H NA disappearance after H-tyrosine administration is more appropriate because it measures the ³H NA formed intraneuronally and it represents another widely used method for the estimation of catecholamine turnover (*cf* Weiner 1974) After a single i.v injection of the labelled NA precursor the initial increase in radioactive NA probably reflects the actual rate of transmitter synthesis (Sedvall *et al* 1968) After a period of 15–30 min, the tissue content of labelled NA is the net result of liberation, uptake, and metabolism of the amine together with a continuous synthesis of labelled transmitter from various tissue pools of exogenous tyrosine (Persson 1969). The possible limitations of the H-tyrosine method reduces its validity in calculating the actual synthesis rate of NA (Persson and Waldeck 1970), but a general consensus seem to exist that it is reliable for comparative purposes (Weiner 1974) The rising H NA activity [illegible] as found in the three of the tissue preparations studied even 4 h after H tyrosi

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the NA content have been reported (Owman *et al* 1975). The axonal uptake mechanism NA deteriorates (Alm *et al* 1979 b) and the tyrosine hydroxylase activity the rate limi step in the NA synthesis, becomes reduced below th. level of detectability towards te pregnancy (Alm *et al* 1979 a) The appearance of the nerve plexus after silver impregna (Gårdmark *et al* 1971) and in histofluorescence studies (Thorbert *et al.* 1978 b) has su gested that these changes are due to degenerative phenomena. This has been verified electron microscopic studies (Sporrong *et al* 1979) The presently reported finding on no measurable ^{3}H NA in tissues from fetus-containing uterine horns after ^{3}H-tyrosine a ministration is directly in line with this A further support was obtained in the experim with 6-OH DA where the chemical sympathectomy resulted in a reduction of the ^{3}H-N formation of the same magnitude as that found in late pregnancy

Although the NA content in the cervix, like that in the uterus, is reduced to very l levels at the end of pregnancy (Owman *et al* 1975), the adrenergic nerve plexus appears be functionally intact in this part of the uterus (Thorbert *et al* 1978 b). The ^{3}H-NA form capacity at 4 h was found to be clearly higher in pregnant than in non-pregnant anim suggesting an increased *in vivo* synthesis of transmitter In accordance with this, the negati k value (reflecting the turnover rate) was significantly higher in the pregnant animals, a as a consequence of this, the apparent half life shorter in the cervix of pregnant anim (4 h) than in the non-pregnant controls (9 h) One factor that to a high degree influences t turnover rate is the impulse traffic in the neurons (Sedvall *et al* 1968 Costa 1972, Wei 1974) In the light of this it is reasonable to assume that the cervix in late pregnancy may under an increased adrenergic influence It has been shown that neuronal hyperactivity related to decreased transmitter levels (Beley *et al* 1976). Thus a high neuronal activity sociated with a high NA turnover may be one reason for the low NA in the cervix during late pregnancy There are, in addition, other possible explanations to this reductio *e g* increased metabolic breakdown of transmitter or impairment of the intraneuro storage function leading to a decreased transmitter retention.

Nerve-mediated contractility of the cervix has been suggested as an important factor the maintenance of human pregnancy (Bayer 1961). However this idea has not been furth pursued, probably due to the lack of knowledge about the anatomical and functional pec liarities of the adrenergic innervation in this region The present indirect evidence for i creased adrenergic impulse traffic in the cervix in late pregnancy gives new support to t idea that neurogenic mechanisms may be of importance in the maintenance of pregnan *i.e* a sphincter mechanism.

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Effects of indomethacin on regional blood flow in conscious rabbits—a microsphere study

By

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Received 30 August 1978

Abstract

BILL, A. *Effects of indomethacin on regional blood flow in conscious rabbits—a microsphere study.* Acta physiol. scand. 1979 *105* 437–442.

In an attempt to reveal the importance of prostaglandins in the control of regional blood flow 20 mg/kg of indomethacin was given i.v. in conscious resting rabbits. Regional blood flow determinations were made before and 20 min after the injection using the labelled microsphere technique. The blood flow in the stomach wall was reduced by 0.73 ± 0.17 g min^{-1} g^{-1} from a level of 1.64 ± 0.24 g min^{-1}g^{-1}. In the jejunum the corresponding figures were 0.44 ± 0.12 and 1.26 ± 0.17 and in the brain 0.29 ± 0.10 and $1.24 \pm$ 0.18. The blood flow in the liver via the hepatic artery increased by 0.20 ± 0.02 g min^{-1} g^{-1} from a level of 0.11 ± 0.02 g min^{-1}g^{-1}. In the retina there was a reduction in blood flow by 2.75 ± 1.03 mg min^{-1} from a starting level of 13.1 ± 2.3 mg·min^{-1}. In a number of other tissues investigated there were no significant effects of the drug. The results suggest that under resting conditions prostaglandins play a role in the control of blood flow in the gastrointestinal tract, the brain and the retina—tissues which are likely to be rather active under such conditions.

Key words: Indomethacin, prostaglandins, unanesthetized rabbit, regional blood flow

It is well established that prostaglandins have strong direct vasomotor effects, are involved in inflammatory reactions and that ischemia tends to cause release of vasodilating prostaglandins at least in skeletal muscle and the kidneys. But prostaglandins also seem to modulate the effects of autonomic nervous stimulation (Samuelsson and Wennmalm 1971, Hedqvist and Wennmalm 1974). Prostaglandins therefore can be expected to be involved in the control of regional blood flow in many tissues—especially under conditions of distress and high activity.

The physiological role of prostaglandins under normal conditions is not well-defined. Recent evidence suggesting involvement of prostaglandins in vasomotor control has been reported for the kidneys (Owen et al. 1975, Herbaczynska-Cedro and Vane 1973, Lonigro et al. 1973, Alonso, Bandow and Rowe 1974, Beilin and Bhattacharya 1977), brain (Pickard and MacKenzie 1973), retina (Pournaras, Tsacopoulos and Chapuis 1978) and stomach (... 1977, Gerkens et al. 1977). So far most studies on prostaglandin effects were per

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TABLE I Effects of 20 mg/kg b wt. indomethaci on regional blood flow in 15 rabbits, M ± SE

Tissue	Normal flow g min⁻¹ g⁻¹	Flow after indomethacin g min g⁻	Change g min⁻ g⁻	
Heart muscle (left ve tricle)	5.82±0.74	6.07±0.74	0 25±0.33	ns
Diaphragm	0 88±0 08	0.92±0.14	0.038±0.11	ns
Skeletal muscle	0.22±0 03	0.20±0.03	−0.021±0 05	ns
Brain	1.24±0 10	0.95±0.10	−0.29±0.10	p 0 01
Choroid plexus	5 17±0.39	5 41±0.90	0.25±0.87	ns
Adrenals	1.37±0.14	1.52±0.16	0.15±0 09	ns
Kidney cortex	6.35±0 47	6 04±0.52	−0 31±0.37	ns
Stomach wall	1 64±0..4	0.89±0.11	−0.75±0.17	p 0 001
Jejunum	1.26±0.17	0.82±0.12	−0.44±0.12	p 0 01
Liver (hep artery)	0.13±0 02	0 33±0 03	0.20±0.02	p<0 001
Spleen	3.88±0.42	3.89±0.83	0.007±0 69	ns
Gall bladder	2.20±0.26	2.30±0.3	0.10±0.27	ns

formed in isolated preparations or in anesthetized animals. A recent exception (Now and Wennmalm 1978)—a study in unanesthetized man—indicated vasoconstrictive effe of indomethacin in the kidneys and splanchnic area which was interpreted to indicate th blood vessels in these regions are normally under the influence of prostaglandins.

In the present study an attempt was made to reveal a physiological influence of pros glandins on regional blood flow in resting unanesthetized rabbits. Labelled microsphe were used to determine regional blood flow in different tissues before and after administ tion of indomethacin The dose chosen was such as to cause marked inhibition of pros glandin synthesis.

Methods

Albino rabbits of both sexes and weighing 1 9–2.7 kg were employed. One day before the blood f measurements one femoral artery was cannulated with a polyethylene tubing for blood sampling and left heart ventricle was cannulated via the left subclavian artery using another tubing. The tubings w filled with a hepari solution to prevent clotu g. The cannulation was performed ith the animals un pentobarbital anesthesia. On the ne t day the animal was placed in a open box, 1000 IU Heparin injected i. and th blood pressure was measured th ough the tubing placed in the femoral artery C was taken not to upset the animal. About 1 ml of suspension of ⁸⁵Sr-labelled 15 μm microspheres (Company St Paul Minnesota) were injected nto the left heart ventricle over 10–15 From the star the injection and fo the ne t 60 s blood was collected from the tubing in the femoral artery The bl flow was about 1 ml/min and the blood was collected n 10 s samples. The measurement of the blood p sure was then resumed and indomethacin, 20 mg/kg b w was i jected i The indomethacin had been solved in a phosphate b ffer pH 8 0, immediately before the injection. The volume injected was Sonication was used to suspend the indomethacin not dissolved There was little effect of the injection the blood pressure About 20 m later new blood flow measurement was made using ⁵¹Cr-labelled μm microspheres. Each injectate contained bout 1 7 million microspheres. After the second injection spheres the animal was killed by an overdose of pent barbital sodium followed by saturated KCl.

In 5 control expts. 10 ml of the phosphate buffer was injected without indomethacin. Flow determ tions were made as in the expts. with the drug.

Essay

The amount of labelled spheres present in the tissue nd blood samples was determined by two-chan gamma spectrometry and the regional blood flow was calculated as described elsewhere (Stjernschan Alm and Bill 1976).

...e II. Effect of 20 mg/kg b. wt. indomethacin on regional blood flow in the eyes of 10 rabbits, M ± S.E.

...e	Normal blood flow mg/min	Flow after indomethacin mg/min	Change mg/min	
...a	15.1 2.3	12.4 ± 2.4	-2.75 ± 1.03	$p < 0.025$
...eal	1000 63	930 ± 83	-68.9 ± 64.4	ns
	68.2 7.6	72.8 ± 6.3	4.59 ± 7.42	ns
...ry ...ocesses	71.6 5.1	70.5 ± 5.1	-1.10 ± 4.0	ns

Results

...t mean arterial blood pressure just before the first blood flow measurement was 85.3 ± 2.28 ...mHg. 5 min after the injection of indomethacin the mean arterial pressure was 83.8 ± 2.9 ...d about 20 min later just before the second blood flow measurement it was 81.6 ± 1.8 ...mHg.

...able I and II show that indomethacin had no or only small effects in most tissues. There ...were notable exceptions. In the stomach wall there was a marked reduction in the blood ...w and in the liver the hepatic arterial blood flow more than doubled. The blood flow in ...e small intestine was also markedly reduced. The reductions in the cerebral and retinal ...ood flows were small but statistically significant.

...jection of phosphate buffer without indomethacin had no appreciable effect on the ...ood flow in any of the tissues investigated.

Discussion

...e results indicate that in unanesthetized rabbits indomethacin causes a marked redistribu... of the blood flow in the liver and gastrointestinal tract and also reduced the blood ...w in the brain moderately. But in most tissues the effects on the blood flow—if any—...re small.

...domethacin concentrations of less than 25 µg/ml have been reported to abolish the out... of prostaglandins from isolated perfused rabbit hearts (Pham-Huu-Chanh, Junstad ...d Wennmalm 1972) and 20 mg/kg body weight was reported to abolish the release of ...staglandin from the kidney (Herbaczynska-Cedro and Vane 1973). Doses as low as ... mg/kg b. wt. were reported by Olow Lundén and Änggård (1978) to cause marked in...bition of prostaglandin synthesis in rabbit kidneys. The dose of indomethacin used in the ...esent experi., 20 mg/kg b.wt., thus can be expected to have produced a marked reduction ...f prostaglandin production.

...*Gastrointestinal tract.* Gastric mucosal erosions are side effects of nonsteroid antiinflam...atory drugs. Whittle (1977) reported flow measurements in urethane anesthetized rats ...ith acidic saline perfusion of the stomach and observed that indomethacin caused a blood ...ow reduction but had no consistent effect on acid loss or the potential difference across the ...ucosa. It was suggested that microcirculatory regulation by prostaglandins may be more ...levant in pathophysiological conditions. A recent study be Gerkens *et al.* (1977) in dogs

under general anesthesia indicated that indomethacin reduced resting blood flow in the stomach wall but not in the small intestine. Hyperemia produced by injection of pentagastrin was counteracted in the small intestine as well as in the stomach. Acid secretion under these conditions was increased by indomethacin The present results indicate that gastric blood flow is reduced by indomethacin even in resting unanesthetized animals and that flow in the small intestine is reduced as well

In the experiments by Nowak and Wennmalm (1978) there was a reduction in splanchnic blood flow as determined from the elimination in the liver of indocyanine green dye. Total liver blood flow thus was reduced in their expts.

Since labelled microspheres are caught in the first capillary bed through which they pass liver blood flow measured with microspheres is essentially hepatic artery blood flow. The rather marked increase in flow observed may be an effect of reduced prostaglandin production in the walls of the hepatic resistance vessels or it may be secondary to a reduction in blood flow through the stomach and the small intestine The latter alternative seems more likely since it is well known that hepatic artery flow tends to increase when portal venous flow is reduced (see Bradley 1963) and at least in dogs PGA and PGA tend to reduce vascular resistance in the hepatic artery and its branches (Hansson and Post 1974)

Kidney Several studies on the effects of indomethacin in anesthetized animals (Owen et al 1975 Herbaczynska-Cedro and Vane 1973 Lonigro *et al* 1973) suggested that in the kidney prostaglandins are continuously involved in the control of blood flow A report by S... *et al* (1975) indicated however that in unanesthetized dogs with an undisturbed renal circulation indomethacin had practically no effect on the renal blood flow A marked effect on reactive hyperemia after short lasting ischemia was observed indicating that prostaglandins probably play a role under certain conditions such as anesthesia and hemorrhage. But the possibility of species differences and regional effects in the kidney also has to be considered. In a recent study bei Beilin and Bhattacharya (1977) in unanesthetized rabbits indomethacin tended to reduce renal blood flow but the effect was marked only in the inner cortex. In the experiments by Nowak and Wennmalm (1978) in unanesthetized man also indicated renal vasoconstriction after indomethacin—an effects that was overcome by infusion of PGE In the present expts. the cortex was not divided into different parts, the samples containing mainly middle cortex. As in Beilin and Bhattacharya s study there was no statistically significant effect in this region

The eye Postaglandins injected into the anterior chamber have very marked effects on the blood vessels of the anterior uvea, causing vasodilatation and capillary damage even in low concentrations (Eakins 1977) The lack of effect of indomethacin in the anterior uvea indicates that there is no significant liberation of vasoactive prostaglandins in this tissue under normal conditions In the retina there was a slight reduction in flow suggesting that significant amounts of prostaglandins may be released in this tissue. A small effect of indomethacin on the calibre of retinal vessels has recently been observed also by Pournaras, Tsacopoulos and Chapius (1978). These results support the hypothesis of Bito (1977) that the physiological role of a postaglandin accumulating mechanism present in the ciliary processes is to remove prostaglandins diffusing forward from the retina and threatening the anterior uvea.

The brain. In a previous study with close arterial infusion of indomethacin in anesthetized [illegible] Pickard and Mackenzie (1973) observed a 38% reduction in cerebral blood flow [illegible] a baseline value of 0.57 ml min^{-1} g^{-1} tissue but there was no effect on the oxygen [illegible]sumption. The response of the cerebral vessels to hypercapnia was markedly reduced. [illegible] the present expts. the reduction in blood flow was rather small and variable but the [illegible] support the hypothesis that prostaglandins may be involved in the control of cerebral [illegible] flow

[illegible] Recent reports suggest that indomethacin affects resting blood flow in skeletal [illegible] very little but that reactive hyperemia and hyperemia during work is markedly [illegible] after indomethacin pretreatment (Kilbom and Wennmalm 1976, Messina, Weiner [illegible] Kaley 1977). Indomethacin has been reported to reduce coronary blood flow in isolated [illegible] preparations from guinea pigs (Schrör, Krebs and Nookhwun 1976) but to have no [illegible] effect on coronary blood flow isolated rabbit hearts (Needleman *et al.* 1975). [illegible] of hypoxia were reduced in dogs under general anesthesia (Afonso, Bandow and [illegible] 1974) but no such effect could be demonstrated in anoxic rabbit hearts (Block *et al.* [illegible]). In the present study indomethacin affected neither skeletal muscle blood flow nor [illegible] perfusion in a detectable way

One can speculate on the basis of previous and the present results that prostaglandin [illegible] is related to the activity of the tissues and tends to increase in anesthesia and due to [illegible]. In the present study the lack of effect of indomethacin on most tissues may have [illegible] due to the resting state of the animals. The effects on the gastrointestinal tract may [illegible] a relatively high activity in this region as can be expected under the conditions of the [illegible]

Indomethacin may affect vascular tone in other ways than by inhibiting prostaglandin [illegible] (Northover 1977). Therefore more definite conclusions concerning the physio-[illegible] role of prostaglandins in the control of vascular tone will have to be based on further [illegible] with various other agents inhibiting prostaglandin synthesis or action.

[illegible] was supported by grant B78-14X-00147 14B from the Swedish Medical Research Council [illegible] EY 00475 from the National Eye Institute, US Public Health Service. I would like to thank [illegible] Theris and Miss Maneta Lindk[illegible] for valuable technical assistance.

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Acta physiol. scand. 1979. 105. 443–452

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Effects of 4-aminopyridine on the excitation-contraction coupling in frog and rat skeletal muscle

By

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Abstract

[illegible] A. R. and K. A. P. EDMAN. *Effects of 4-aminopyridine on the excitation-contraction coupling in frog and rat muscle.* Acta physiol. scand. 1979. *105.* 443–452.

[illegible] effects of 4-aminopyridine (4-AP) were studied on isolated single muscle fibres of the frog and toe [illegible] of the rat. In both muscle preparations, 4-AP potentiated the twitch amplitude [illegible] without significantly affecting the tetanus response. There [illegible] an increase of the time to peak tension and, in frog muscle, [illegible] time to half relaxation. 4-AP produced no change of the resting membrane potential. The rate [illegible] and, hence, the total duration of the action potential [illegible] were markedly prolonged. 4-AP did not [illegible] contractures by itself nor did it affect the contracture induced by caffeine. The mechanical threshold [illegible] by measuring the contracture response to various degrees of depolarization by potassium. [illegible] threshold was not affected by 4-AP. T[illegible] twitch potentiation by 4-AP [illegible] independent of the extracellular [illegible] concentration. [illegible] is concluded that 4-AP potentiates the twitch response by increasing the release [illegible] calcium into the myofibrillar space by prolongation of the action potential. In addition, there [illegible] more direct inhibitory action of 4-AP on the calcium re-uptake by the sarcoplasmic reticulum in [illegible]

[illegible] 4-aminopyridine, skeletal muscle, single muscle fibre, excitation-contraction coupling, contrac[illegible], action potential, membrane potential

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[illegible] interest has been focused on the muscular effects of 4-aminopyridine (4-AP), [illegible] which has been show[illegible] to counteract the paralysis caused by curare (Paskov *et al.* [illegible]). The action of 4-AP on neuromuscular junction has been studied and there is evidence [illegible] this agent greatly potentiates transmitter release possibly by increasing the level of Ca [illegible] the nerve terminal (Molgo *et al.* 1975, 1977, Lundh and Thesleff 1977). However [illegible] is known as to whether or not 4-AP also affects the contractile performance by acting [illegible] on the excitation-contraction coupling. This latter aspect has been elucidated in [illegible] detail in the present study. To this end the effects of 4-AP on the time course of twitch [illegible] tetanus have been analyzed on single muscle fibres of the frog and on curarized toe [illegible] of the rat. Evidence will be presented to show that the twitch potentiation produced [illegible] 4-AP may at least partly be accounted for by prolongation of the membrane action [illegible]

Methods

Preparation

Frog muscle Single muscle f bres were isolated from the ventral head of the semitendinosus muscle *Rana temporaria*. For mounting the fibres, a link of stainless steel wire (thickness 0.1 mm) was attached each tendon as described previously (Edman and Kiessling 1971).

Rat toe muscle Thin toe muscles were dissected from the front legs of Sprague-Dawley rats (150-200 A small loop of silk thread was tied to the tendon at each end of the muscle.

M scle chamber

The preparations (frog single fibres or rat whole muscles) were mounted horizontally in Perpex cham between tension transducer (see below) and an adjustable stainless steel hook, the position of hich co be set by means f a micrometer screw The chamber was 6 mm wide, 5 mm deep and contained 12 solution. The solution was changed by introducing fresh solution at the transducer end of the chamb and it was removed by suction drain t the other end A flush time of about 3 s as used hen the pot sium solutions were added for production of contractures. This caused an almost complete (95 %) change of the bathing solution (Andersso and Edman 1974 b).

T mperature ontrol

During an experiment the temperature was controlled by a Colora Ultra-thermostat hich circulated ethylene glycol-water mixture through jackets surrounding the chamber and the containers of solut The solution containers were connected with the ch mber through polyethylene tubes (approxim 10 cm long) and a stopcock at the chamber inlet.

In studies on single muscle f bres, the bath temperat re varied between 1 and 3.5°C and in studies rat toe muscles between 22 and 23 C from expt. to expt The temperature as maintained constant within ±0._°C throughout ny particular expt., even during exchange of solution.

Tension recording

Tension was recorded by means of an RCA 5734 mechano-electric transducer The signals were displa on a Tektronl 50 A oscilloscope and were recorded simultaneously on paper by means of an Elen Schönander ink-jet recorder

Electrical stimulation

The single muscle fibre or the rat toe muscle was stimulated by means of a multielectrode assembly described previously (Edman and Kiessll g 1971) Pulses of 0._ ms duration were used and the stimulus strength was adjusted to be supramaximal fo each lectrode pair For tetanic stimulation, 1 s train pulses with a frequency of 16–20 Hz was used for the si gle muscle f bre To obtain a fused tetanus in t toe muscle, 1 s train of pulses with a frequency of 100 Hz was used.

The single muscle f bre or the rat toe muscle was mounted for t least 1 h before the expt. and tetanized at 10 min tervals d ring this time. During the actual expt. the preparation was stimula every 2 mi (if not otherwise stated) to record either twitch tetanus response.

Solutions

Frog muscle fibre experiments The solutions used had the following composition (mM)

Ordinary Ri ger sol tion NaCl 115 5 KCl _0, $CaCl_2$ 1.8, Na-phosphate buffer 2.0, pH 7.0.

Calcium-free Ringer NaCl 116.3 KCl 2.0, EDTA 1.0, Na-phosphate buffer 2.0, pH 7.0.

Lanthanum conta ning Ringer NaCl 117.2, KCl 2.0 La 0.05 Tris-(hydroxymethyl)-aminometh 2.0. The pH was adjusted to 7.0 by addition of H_2SO t a final concentration of 0.94 mM.

For potassium contractures, solutions of the following composition were used

Solution (mM)	KCl	K CH_3SO	Na-CH_3SO	NaCl	$CaCl_2$
10 K Ringer	10	—	96.90	10.60	1.80
20 K Ringer	—	20	88.99	8.51	1.80
40 K Ringer	—	40	75.05	2.45	1.80
80 K Ringer	—	80	37.80	—	1.50
117.5 K Ringer	—	117.5	0.77	—	1.03

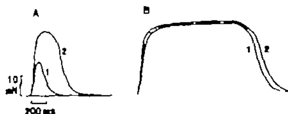


Fig. 1. Effects of 4-AP on isometric twitch (A) and tetanus (B) in frog single muscle fibre. 1. Control, normal Ringer solution. 2. In the presence of 3 mM 4-AP. Stimulus markers below base line.

[illegible] Ringer solutions contained 2 mM Tris-(hydroxymethyl)-aminomethane. The pH was adjusted to [illegible] by addition of H_2SO_4 to [illegible] final concentration of 0.94 mM.

[illegible] muscle experiments, Tyrode solution (mM): NaCl 154.0, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 1.0, [illegible]PO_4 [illegible], Na_2HPO_4 [illegible], glucose 5.5, 0.5 mg/ml of d-tubocurarine. This solution was aerated by [illegible] of [illegible] O_2 and 5 [illegible] CO_2.

Drugs 4-aminopyridine (4-AP), caffeine and dantrolene [illegible] were dissolved in Ringer and Tyrode solutions, [illegible].

[illegible] contractures in frog muscle fibres

[illegible] of the following concentrations were used (mM): 1.0, 1.5, [illegible], 3.0 and 5.0. The solutions [illegible] from [illegible] pre-cooled container until plateau contracture tension [illegible] achieved. This occurred [illegible] approximately 15 [illegible]. Relaxation was produced by re-introduction of the normal Ringer solution. The [illegible] interval between two successive potassium or caffeine contractures was 20–25 min.

[illegible] of membrane potential

[illegible] glass capillary electrodes (about 20 MΩ) filled with 2.5 M KCl were used for intracellular [illegible] of membrane potentials. The reference electrode was an Ag-AgCl electrode connected to the [illegible] through an agar-Ringer bridge. The microelectrode and the reference electrode were connected via [illegible] provided [illegible] capacitance neutralization to [illegible] Tektronix 502A oscilloscope. The signals [illegible] photographed on 35 mm film. The amplified [illegible] signals were also used to modulate an audio frequency [illegible] successful impalement [illegible] as indicated by an abrupt change in frequency. When action potentials [illegible] recorded the fibre was stimulated only at one locus, approximately 5 mm from the tip of the microelectrode.

Results

[illegible] Frog muscle fibres

Twitch and tetanus responses. 4-aminopyridine (4-AP) was tested on isolated muscle fibres [illegible] concentrations ranging between 0.02 and 3.0 mM. In a concentration of 1 mM or higher [illegible] 4-AP potentiated the twitch response without significantly affecting the tetanus response. [illegible] twitch potentiation was obtained by 3 mM 4-AP (Fig. 1). The magnitude of the twitch potentiation [illegible] inversely related to the twitch/tetanus ratio recorded in the control Ringer solution. At full effect of 4-AP a peak twitch force of more than 90% of the tetanic tension was generally attained.

As illustrated in Fig. 1, 4-AP caused no significant change in the initial rate of rise of [illegible]. The increase in twitch amplitude was associated with an increase of the time to peak [illegible] and a prolongation of the relaxation phase. With 3 mM 4-AP the half time to peak [illegible] and the half time for relaxation increased by 95 [illegible] and 74% of the control values, respectively (Table 1).

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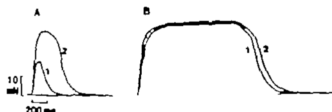
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TABLE 1 Effects of 4-AP on twitch parameters in frog single muscle fibres. Each value represents the m[illegible] of 5–10 twitch responses.

Expt. no	Normal Ringer		3 mM 4-AP in Ringer		T_A/T_C	R_A/R_C
	Half time to peak tension (ms) T_C	Half time for relaxation (ms) R_C	Half time to peak tension (ms) T_A	Half time for relaxation (ms) R_A		
1	85	130	14	210	1.67	[illegible]
2	86	144	180	63	2.09	[illegible]
3	85	165	177	[illegible]	[illegible]	[illegible]
4	76	17	148	[illegible]	1.95	[illegible]
5	69	84	136	184	1.97	[illegible]
Grand mean	80	139	157	234	1.95	[illegible]

The effect produced by 4-AP was only partially reversible. As illustrated in Fig. [illegible] peated washing of the fibre in normal Ringer lowered the amplitude of the potent[illegible] twitch slightly. When 3 mM 4-AP was re-introduced the twitch amplitude was agai[illegible] creased.

To test if the extracellular calcium plays any role in the twitch potentiation produc[illegible] 4-AP, calcium was removed from the extracellular medium by repeated washing of the f[illegible] with a calcium free Ringer solution containing 1 mM EDTA. Fig. 3 b shows, in confir[illegible] tion of previous results (Edman and Grieve 1961, 1964, Jenden and Reger 1963, C[illegible] 1963), that removal of calcium from the extracellular medium made the fibre inexcit[illegible] Addition of lanthanum to the bathing solution in a concentration (0.05 mM) sufficie[illegible] normalize the resting membrane potential in the absence of calcium (Andersson and Ed[illegible] 1974 a) restored the twitch response (Fig. 3 c). 4-AP (3 mM) added to the calcium- lanthanum-containing medium caused a potentiation of the twitch in much the same as in normal Ringer (Fig. 3 d).

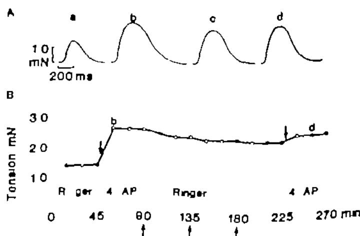


Fig. 2. Reversibility of the effects produced by 4-AP on the twitch response of frog muscle fibre. Tra[illegible] in A refer to times indicated in B. Arrows above curve in B indicate addition of 3 mM 4-AP. Arrows b[illegible] abscissa indicate washings with Ringer solution. Note that twitch potentiation is only partially rever[illegible]

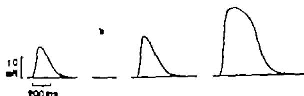
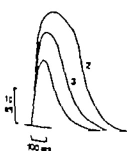


Fig. 3 Effect of 4-AP on twitch response of frog muscle fibre after removal of calcium from the extracellular solution (a) Normal Ringer (b) Approximately 5 min after removal of calcium (1 mM EDTA added). No twitch response. (c) After replacing calcium with 0 05 mM lanthanum. (d) Effects of 3 mM 4-AP calcium free Ringer containing 0 05 mM lanthanum.

Dantrolene has a powerful depressant effect on the isometric twitch. In relatively low concentrations, it greatly suppresses the twitch response of single muscle fibres (Ellis and [illegible] 1972, Hainaut and Desmedt 1974). The effect of dantrolene on 4-AP-treated fibres was studied. As illustrated in Fig. 4 dantrolene reduced the 4-AP potentiated twitch.

Potassium and caffeine induced contractures Experiments were performed to find out if 4-AP affected the mechanical threshold, *i* [illegible] the membrane potential at which contractile activation is initiated. For this purpose contractures were produced by immersing the single fibre in an isotonic solution containing potassium (constant [K]. [Cl] product) in concentrations varying between 10 and 117.5 mM. Peak contracture tension was plotted against log [K]. Fig. 5 summarizes the results obtained in three experiments, in which the effect of 3 mM 4-AP was tested. There was a very steep rise of the contracture response in the range [illegible]-20 mM potassium. As can be seen, 4-AP did not cause any significant change of the fibre's response to potassium depolarization.

Similarly the effect of 4-AP on caffeine induced contractures was studied. The peak tension developed was plotted against log caffeine concentration. The S-shaped curve relating contracture tension and caffeine concentration also remained unaltered in the presence of 3 mM 4-AP (Fig. 6).



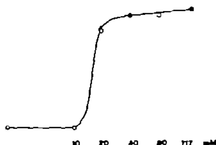


Fig 4

Fig 5

Fig. 4 Effects of dantrolene on the twitch response of frog muscle fibre in the presence of 3 mM 4-AP 1. Normal Ringer 2. In the presence of 4-AP 3. In the presence of 4-AP and 9 μM dantrolene.

Fig. 5 Relation between peak contracture tension and extracellular potassium concentration in the presence and absence of 4-AP. Frog muscle fibres. O Control, no 4-AP △ In the presence of 3 mM 4-AP. Each point represents mean value of three different experiments.

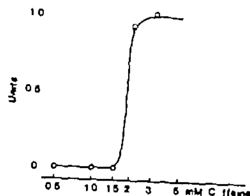


Fig. 6



Fig. 7

Fig. 6. Relation between peak contracture tension and caffeine concentration in the presence and ab of 4-AP Frog muscle f bres. O Control, no 4-AP Δ: In the presence of 3 mM 4-AP Each point repr mean value of three d fferent experiments.

Fig. 7 Effects of 4-AP on intracellularly recorded action potential of frog single muscle fibres. A. No Ringer B In the presence of 3 mM 4-AP The records in A and B are from different fibres.

Resting and action potentials 4-AP in a bath concentration of 3 mM did not affect resting membrane potential over at least 2 hours as tested in 13 fibres of altogether 6 fi bundles (Table II) The effects of 4-AP on membrane action potential were studied after twitch potentiation had developed. There were no significant changes of the maximum r of rise and overshoot of the action potential The maximum rate of fall and the durat of the action potential, on the other hand were both markedly prolonged (Fig 7 a Table II)

B *Rat toe muscle*

Experiments were performed to study the effects of 4-AP on twitch and tetanus respo of mammalian skeletal muscle Curarized toe muscles of the rat were stimulated at 22 23 to produce a single twitch or a 1 s fused tetanus at 2 min intervals. 4-AP in a concentrat >0 1 mM caused potentiation of the isometric twitch (Fig. 8). The peak twitch amplit was increased by approximately 1/3 of the control in response to 0 1 mM 4-AP Similar to t situation in frog skeletal muscle 4-AP prolonged the time to peak twitch tension. Howev

TABLE II Effects on resting and action potentials of 3 mM 4-AP added to normal Ringer (Mean ± S Frog muscle f bre bundles exposed to 4-AP for 15–20 min before recording the resting a action potentials. Number of f bres for each measurement given within brackets Stude t-test. + + + P < 0 001

	Resting potential mV	Action potential			
		Overshoot, mV	Maximum rate of rise, V/s	Maximum rate of decay V/s	Duration at −25 mV leve ms
Control	84.1 ± 2.3 (13)	33 7 ± 2.2 (10)	56.0 ± 3.5 (10)	26 4 ± 1.8 (10) + + +	5.2 ± 0 2 (10) + + +
4-AP (3 mM)	87 6 ± 1.8 (13)	25.5 ± 3 3 (7)	55.9 ± 4 6 (7)	4 3 ± 0.4 (7)	16.2 ± 1.6 (7)



[illegible] Effects of 4-AP on isometric twitch (A) and tetanus (B) of curarized toe muscle of the rat. 1 [illegible] Tyrode solution. 2. In the presence of 0.1 mM 4-AP Note different time and tension scales.

[illegible] time to 50% relaxation in the rat toe muscle was not significantly affected (Table III) [illegible] was no clear effect of 4-AP on the amplitude of the fused tetanus when care was [illegible] to stimulate the muscle supramaximally When submaximal stimulation strengths [illegible] used, 4-AP in concentrations >0.1 mM reduced the tetanic force. This suggests that [illegible] raised the electrical threshold thereby reducing the number of fibres activated under [illegible] conditions.

Discussion

[illegible] experiments (Lundh *et al* 1977) performed on the rat extensor digitorum longus [illegible] suggest that 4-amino-pyridine (4-AP) potentiates muscle contraction by facilitating [illegible] release at the neuromuscular junction and the drug has been shown to be a [illegible] antagonist of the neuromuscular blocking agent botulinum toxin (Lundh *et al* [illegible], Lundh and Thesleff 1977). The present results on frog single muscle fibres and on [illegible] rat toe muscles demonstrate that 4-AP causes twitch potentiation by a more [illegible] action on the excitation-contraction coupling. In order to produce this effect, however, considerably higher concentrations were required than those needed to affect the [illegible] release (Lundh 1978).

The twitch potentiation by 4-AP is characterized by an increased peak amplitude and an [illegible] of the half time to peak tension and of the half time for relaxation. These changes [illegible] associated with a broadening of the action potential. As previously demonstrated on [illegible] muscle fibres (Edman *et al* 1966) and in experiments on whole muscle (Sandow and [illegible] 1964, Sandow Taylor and Preiser 1965 Taylor *et al.* 1972) the duration of the [illegible] activity appears to be quantitatively related to the action potential duration [illegible] 25 mV level). This has been suggested to mean, that the action potential governs

[illegible] Effects of 4-AP (0.1 mM) on the twitch parameter of curarized toe muscles of rat. Each value represents the mean of 3–4 responses.

Exp. no.	Normal Ringer		3 mM 4-AP in Ringer		T_A/T	R_A/R_O
	Half time to peak tension (ms) T_O	Half time for relaxation (ms) R_O	Half time to peak tension (ms) T_A	Half time for relaxation (ms) R_A		
[illegible]	14	30	19	29	1.36	1 03
[illegible]	12	17	14	17	1.17	1.00
[illegible]	15	23	16	20	1.07	0.87
[illegible] mean	14	23	16	22	1.20	0 97

the time during which activator Ca is released into the myofibrillar space (Sandow et al 1964 Edman et al 1966, Taylor et al 1972) The longer duration of the action potential would cause a higher peak concentration of Ca at the contractile sites, and this in turn would require a longer time for the elimination of Ca from the myofibrils. If the peak concentration of the activator Ca were large enough to fully activate the contractile system, a further increase of the Ca concentration would merely cause a prolongation of the mechanical activity This would be reflected in the isometric twitch response as an increased peak amplitude and a later attainment of peak tension, whereas the initial rate of rise of the twitch tension would remain unaffected. The effects produced by 4-AP are fully consistent with the idea that twitch potentiation is due to the prolongation of the action potential according to the above mechanism However the results clearly show that 4-AP also prolongs the relaxation time in frog muscle suggesting that 4-AP exerts a more direct inhibitory action on the resequestration of calcium in this tissue.

It is of interest to note in this connection that 4-AP does not affect the mechanical threshold, *i.e* the potential level at which contraction is initiated in response to membrane depolarization. This is inferred from the finding that 4-AP does not shift the curve relating potassium concentration and contracture response (Fig. 5).

Caffeine and 4-AP affect the time course of the isometric twitch in a similar way. Previously it was considered that the effect of caffeine is due to an inhibitory action on the calcium pump of the sarcoplasmic reticulum (SR) (Weber 1968) This view has recently been revised by Endo (1975) who suggests that caffeine potentiates the twitch response, and induces contractures in higher concentrations, by enhancing the calcium-induced calcium release from the SR Such a mechanism of action does not seem to operate in twitch potentiation by 4-AP as this agent, even in high concentrations (10 mM), fails to induce caffeine-contractures and has no detectable influence on the contractures induced by caffeine (Fig

From the results presented in Table II it is evident that 4-AP does not cause any detectable change of the resting membrane potential The maximum rate of rise and the overshoot of the action potential were not significantly different from the control. The rate of decay of the action potential on the other hand was markedly reduced. These changes of the action potential are consistent with previous observations which suggest that 4-AP acts by predominantly decreasing the potassium conductance in frog skeletal muscle fibres (Gillespie and Hutter 1975) and in cockroach (Pelhate and Pichon 1974) and squid (... *et al* 1976) axon membranes and in sympathetic nerves (Kirpekar *et al* 1977).

In contrast to its effects on the nerve terminal (Lundh *et al* 1977) 4-AP had a slow onset of action on the muscle fibres, 15–20 min being required for the full effect to appear. Furthermore the effects produced by 4-AP on the action potential and on the twitch were only partially reversible. This is consistent with the idea that there is a very firm binding of 4-AP both to the surface membrane and, in frog muscle, to the sarcoplasmic reticulum inside the fibre (*cf* above).

It has been suggested that 4-AP increases transmitter release from nerve endings by facilitating calcium influx from the extracellular medium (Molgo *et al* 1977 Lundh and Thesleff 1977). The present results suggest, however that extracellular calcium is immaterial for the twitch potentiation produced by 4-AP The enhancement of the isometric tw

[illegible] 4-AP could thus be produced when there was no calcium in the extracellular medium, [illegible] the replacement of calcium in the Ringer solution by lanthanum. It has previously [illegible] shown that the presence of lanthanum (Andersson and Edman 1974 a) prevents the [illegible] depolarization that otherwise occurs as calcium is removed from the bathing [illegible] (Edman and Grieve 1961 1964, Jenden and Reger 1963 Curtis 1963). Lanthanum is [illegible] to penetrate the fibre membrane (Laszlo *et al* 1952, Lesseps 1967 Langer and [illegible] 1972) and causes by itself only a slight twitch potentiation (Andersson and Edman [illegible] in the low concentrations (0 05 mM) used here.

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Studies on the enzymatic blood-brain barrier: Quantitative measurements of DOPA decarboxylase in the wall of microvessels as related to the parenchyma in various CNS regions

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Abstract

Hardebo, J. E., B. Falck, Ch. Owman and E. Rosengren. *Studies on the enzymatic blood-brain barrier: Quantitative measurements of DOPA decarboxylase in the wall of microvessels as related to the parenchyma in various CNS regions.* Acta physiol. scand. 1979. 105. 453–460.

The presence of DOPA decarboxylase in cerebral microvessels (capillaries and venules) impedes the passage of [illegible] precursors into the brain. The relative amount of DOPA decarboxylase in this trapping [illegible] as compared to the parenchyma *per se* was estimated in various CNS regions, measuring the formation of dopamine from L-DOPA *in vivo* or *in vitro* in two experimental models on rats and rabbits. (1) On the basis of the treatment with peripheral decarboxylase inhibitor (carbidopa, which inhibits also [illegible] DOPA decarboxylase in the CNS) it could be calculated that the enzyme in the microvessels of the caudate nucleus (rich in catecholamine nerve terminals), cerebellum (poor in catecholamine nerves), and spinal cord comprised 23, 91 and 79 per cent, respectively, of the total enzyme activity. (2) Measurement of dopamine formation in the spinal cord following transection at the midthoracic level (which causes degeneration of the catecholamine neurons caudal to the lesion since they are all descending) indicated that the same as found in the carbidopa model, 71 per cent, of the total tissue decarboxylase activity [illegible] the microvessel walls. The results show that a considerable portion of tissue decarboxylase in the [illegible] present in the microvessel walls, where it represents part of an enzymatic blood-brain barrier mechanism.

The concept of a barrier between the blood and the brain parenchyma originates from the [illegible] by Ehrlich (1885) that vital dyes—when injected systemically—pass freely from the circulation into peripheral tissues, but are excluded from the central nervous system. With the advent of electron microscopy an ultrastructural basis for the blood-brain barrier was [illegible] in that cerebral vessels are distinguished by the presence of closed tight junctions between adjacent endothelial cells (Reese and Karnovsky 1967). This feature—unique for the cerebral vasculature—along with absence of endothelial fenestrae and the paucity of pinocytotic vesicular transport [illegible] endothelial cells, constitutes the morphological basis

for the blood-brain barrier In this way the diffusion of substances from the systemic circu– tion into the brain is severely limited (for review see Rapoport 1976).

Using fluorometric assay and fluorescence microscopic techniques, a number of hi– chemical investigations have revealed that there is a specific trapping mechanism loc within the cerebral capillary endothelium for amine precursors, such as L DOPA (L dihydroxyphenylalanine) and L 5-hydroxytryptophan (Bertler *et al* 1966, Owman Rosengren 1967 Bartholini *et al* 1971 Hardebo *et al* 1976). This trapping mechan which constitutes part of an enzymatic barrier has essentially three phases the ac transport (Wade and Katzman 1975) of the precursor into the endothelial cell the decarb ylation of the precursor to the corresponding amine and finally the amines are metabol by the enzyme monoamine oxidase

The degree of decarboxylation capacity can be measured directly in isolated fraction brain microvessels which are essentially free of neuronal contamination (Hardebo *et* 1977 a). The capacity of the enzymatic blood brain barrier under *in vivo* conditions has b estimated in terms of the break through of L DOPA into the brain after systemic adminis tion of increasing doses of the amino acid (Hardebo *et al* 1977 b) The present paper d further quantitative aspects of the microvascular trapping mechanism for L DOPA in brain by chemical determinations of the DOPA decarboxylase activity in various CNS reg using two models either after elimination of the neurons (caudal to a spinal cord tran tion) or following selective inhibition of the enzyme in the vessels (by carbidopa which certain dose levels, efficiently blocks DOPA decarboxylase in peripheral tissue and b microvessels, leaving the enzyme within the brain parenchyma essentially unaffec Bartholini and Pletscher 1969)

Material and Methods

Animals The study was performed on 16 female Sprague-Dawley rats, weighing 190–200 g, and 18 fe albino rabbits weighi g 1.5 t 2.0 kg The an mals had free access to standard pellet food and tap ater spinal cord in 11 of the rats and in all rabbit was transected in the midthoracic region under brietal so anesthesia The rinary bladder was emptied da ly by gentle pressure on the bdomen, and care as t to prevent deformation of the extremities. The animals were killed under l ght ether anesthesia 10 days transection by perfusion of the vascula system w th 0 9 saline.

Fluorescence microscop Immediately fter decapitation, small tissue pieces were dissected out fro cerebella hemispheres, the caudat ucleus, and the spinal cord. They were frozen to the temperatu liquid nitrogen and further processed fo monoamine fluorescence histochemistry accord ng to the F Hillarp method (Björklund Falck nd Owma 197). The paraformaldehyde used had previously equilibrated with a r of 70 of humidity The formaldehyde-induced, histochemically rable fluoroph of noradrenaline (NA), dopamine (DA), and DOPA are ndistinguishable under standard condition they have the same spectral characteristics and all exhibit a green light under the optical conditions (Björklund *et al* 1972).

Chemical determination of catecholamines. DA was determined fluorometrically either accordi Häggendal (1963) or Ant n and Sayre (1964) followi g homogenization of the tissue in 0.4 N perch acid. NA was estimated by the radioenzymatic method described by Coello, Haley and I ersen (197 mod fied by Coyle and Henry (1973) the tissue was homogenized n 0.1 N perchloric acid containing 0 ml EDTA.

Determination of DOPA decarboxylase activity (1) One mm thi slices of the cerebellum, caudate and cervical spinal cord were cut with a razor blade and transferred t incubation ials containing ice Krebs-Ringer buffer solution. The buffer had the f llowing composition (mM) NaCl 118, KCl 4.5, Ca $2H_2O$ 2.5 MgSO $7H_2O$ 1.0, $NaHCO_3$ 25 KH PO 1.0 t which was added 1 mg/ml glucose, 0.2 m ascorbic acid and 0.1 mg/ml EDTA. The vials were placed in n incubation bath t 37°C for preincubati

...g) and followed by incubation during 20 min in the presence of 0.1 mg/ml L-DOPA. Blanks were ... without L-DOPA. The buffer solution was continuously aerated with a mixture of 95% O₂ and 5% ... pH of 7.4. One slice from each incubation was taken for fluorescence microscopy and the re... for chemical determination of DA according to Hällgrendal (1963).

...) Tissue pieces from the spinal cord were homogenized on ice and preincubated for 10 min in 67 mM ... phosphate buffer (pH 7.5) to which pyridoxal-5-phosphate had been added, followed by incubation for ... in the presence of 0.1 mg/ml L-DOPA. The incubation was performed at 37 C under anoxic condi... (continuous bubbling with nitrogen, Bertler and Rosengren 1959). The incubation was stopped with ... perchloric acid. Boiled homogenate incubated in L-DOPA served as blank. The amount of DA formed ... determined according to Hällgrendal (1963).

... was no statistically significant difference (Student's t-test) whether the dopamine formation was ... and under anoxic conditions or not, even with extended incubation times from 20 to 45 min. ... therefore not specified in each of the two techniques that was used in the DOPA decarboxylase ... measurement.

Experimental. Three types of experiments were performed with animals in which the spinal cord had been ... sected.

1) Three rats and 8 rabbits were pretreated with the monoamine oxidase inhibitor nialamide (100 mg/kg ...) ... before the injection of L-DOPA (50 mg/kg i.p.). All rats and 6 rabbits were killed 20 min later ... rabbits were killed after 60 min. The spinal cord was dissected out and pieces were taken cranial ... to the lesion for chemical determination of DA.

2) Three rats and 4 rabbits were pretreated with nialamide (100 mg/kg i.p.) 90 min before sacrifice. One ... slices of the spinal cord were taken above and below the level of transection for determination of ... DOPA decarboxylase activity under normoxic conditions.

... Spinal cord tissue above and below the lesion was homogenized on ice for determination of DOPA ... decarboxylase activity under anoxic conditions (3 rats and 4 rabbits) or homogenized in perchloric acid for ... determination of NA (2 rats and 2 rabbits).

Experiments with DOPA decarboxylase inhibition were carried out with intact rats. 5 rats were pretreated ... reserpine (10 mg/kg i.p.) and 4 h later with nialamide (100 mg/kg i.p.), followed after 30 min by carbi... dopa (100 mg/kg i.p.) which effectively inhibits DOPA decarboxylase in peripheral tissue and brain ... vessels, (leaving the decarboxylase activity within the brain parenchyma essentially unaffected, ... Lindvall and Fletcher 1969). The animals were killed 1 h after the last injection. 5 animals received ... reserpine and nialamide, but no carbidopa. Slices from the cerebellum, caudate nucleus, and spinal cord ... were incubated under normoxic conditions for determination of DOPA decarboxylase activity.

Drugs. The following compounds were used: 3,4-dihydroxyphenylalanine (Sigma), reserpine (Serpasil, ...), nialamide (Niamid, Pfizer), α-methyldopa hydrazine (Carbidopa, MSD), pyridoxal-5-phosphate ... Drug House), brietal sodium (Lilly).

Results and Comments

Fluorescence microscopy of the caudate nucleus from animals treated with reserpine and nialamide with or without the peripheral decarboxylase inhibitor carbidopa, followed by ... incubation with L-DOPA showed an intense green fluorescence in the parenchyma deriving from the very dense network of dopamine-containing axon terminals (cf. Lindvall and Björklund 1974). The general background fluorescence of the parenchyma in the cerebellum and spinal cord was very low and only a small number of isolated green-fluorescent axons known to contain NA (Olson and Fuxe 1971, Pickel, Segal and Bloom 1974, Nygren and Olson 1977) was present in the preparations.

In animals not given carbidopa, a green fluorescence of equally high intensity was seen in the walls of capillaries as well as of small veins in all regions studied (Fig. 1), whereas the walls of parenchymal and pial arteries and arterioles were non-fluorescent. The microvessel fluorescence was weaker in the rabbits (killed 20 or 60 min after administration of L-DOPA) than in the rats (killed 20 min after L-DOPA). Tissues from animals pretreated with carbidopa

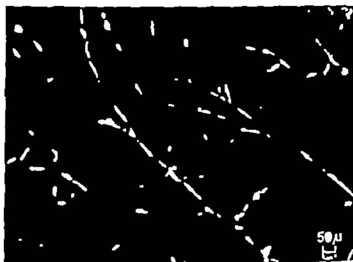


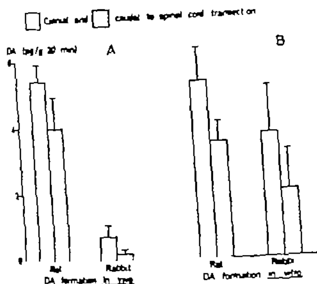
Fig. 1 Spinal cord from a rat pretreated with 10 mg/kg reserpine followed by 100 mg/kg nialamide. 2h after the injection of 50 mg/kg L DOPA an intense green fluorescence is seen in the microvessel walls T is essentially no background fluorescence in the parenchyma. Those of the nuclear regions that are fo the tissue section represent the largest olume of the pericytes and endothelial cells and are therefore pa larly well visible.

(following reserpine and nialamide as above) and incubated with L DOPA showed on weak fluorescence in the microvessel wall but, on the other hand, an increased diffuse b ground fluorescence of the parenchyma

The density of the network of microvessels was compared in the 3 regions on the basi the arrangement of intensely fluorescent vessel walls The density was lower in white tha grey matter but no difference in density was found between the various regions.

Microvessel DOPA decarboxylase activity (carbidopa model) An estimate of the D(decarboxylase activity in caudate cerebellar and spinal cord microvessels was obtaine subtracting the tissue decarboxylase value in animals treated with carbidopa (and thus ha only central neuronal decarboxylase intact) from the value of animals not given carbid (having both neuronal and vascular decarboxylase) Values obtained during incubation w out L DOPA (representing endogenous DA) were subtracted beforehand. The vascular carboxylase activity thus calculated was in the cerebellum and spinal cord 2.82±0 50 3 12±0 83 μg DA/g tissue 20 min, respectively (mean values ± S.E. Table I). In caudate nucleus the mean activity was slightly higher 4 72±0 78 mg DA/g 20 min. relative vascular activity comprised 25 91 and 79 per cent of the total (neuronal vascular) DOPA decarboxylase activity in the caudate nucleus, cerebellum and spinal c respectively Since the cerebellum and spinal cord contain only few catecholamine-form neurons it is to be anticipated that a major fraction of the total decarboxylase activity in t regions is represented by the microvessel walls. The situation is reverse in the caudate nucl rich in dopaminergic nerve terminals.

In untreated animals the NA concentration in the spinal cord is about the same in segm cranial and caudal to the midthoracic level (Andén, Magnusson and Rosengren 1965). days after thoracic transection the concentration caudal to the lesion dropped to 13±2 11±2 per cent of the concentration cranial to the section in the rat and rabbit, respecti



DOPA decarboxylase activity (mean values ± S.E.) expressed as µg dopamine (DA) formed per g spinal cord tissue (wet weight) cranial and caudal to cord transection in the rat and rabbit. A. Formation in vivo, animals treated with nialamide (100 mg/kg i.p.) 1 h followed by L-DOPA (50 mg/kg i.p.) 20 min. B. Formation in vitro, tissue slices preincubated in nialamide (10^{-4} M) 20 min, followed by incubation in DOPA (0.1 mg/ml) 20 min.

...cranial DOPA decarboxylase activity (spinal cord transection model). The degree of DA formation obtained in vivo and in vitro cranial and caudal to the midthoracic transection in the rat and rabbit are shown in Fig. —. In non-operated animals, the DA formation is about the same cranial and caudal to the transection level (Andén et al. 1965). However after transection the decarboxylase activity in the cranial portion does not differ significantly from the activity in the corresponding region of non-operated animals (Andén et al. 1965). In the present study a mean fall in activity of 29 per cent was seen in the rat after transection, i.e. the DOPA decarboxylase activity present in microvessel walls (and in perivascular sympathetic nerves) could constitute about 70 per cent of the total activity in the spinal cord (cf. the value...

Table 1. DOPA decarboxylase activity in various CNS regions of the rat separately expressed in terms of enzyme activity in the parenchyma (neuronal) and in the microvessel walls

Enzyme activity / CNS Region	A: total activity ("neuronal + microvascular")	B: activity after carbidopa ("neuronal")	A minus B ("microvascular activity")
Caudate nucleus	18.91 ± 1.78	14.19 ± 1.55	4.72 ± 0.78
Cerebellum	3.10 ± 0.46	0.28 ± 0.04	2.82 ± 0.50
Spinal cord	3.95 ± 0.45	0.83 ± 0.23	3.12 ± 0.83

The decarboxylase activity is expressed as the amount of dopamine synthesized in tissue slices (µg formed per g tissue during 20 min) from animals pretreated with reserpine (10 mg/kg i.p.) and nialamide (100 mg/kg i.p.), with or without inhibition of vascular decarboxylase by carbidopa (100 mg/kg i.p.). Slices incubated in the absence of DOPA reflect the level of endogenous dopamine: these values have been subtracted in the calculations of neuronal and microvascular decarboxylase activity. Figures show mean values ± S.E.M., number of animals 3.

calculated after vascular decarboxylase inhibition) The corresponding value in the rat was considerably lower about 45 per cent.

Discussion

The mechanism that involves decarboxylation and subsequent degradation of circulating monoamine precursors at the level of the blood brain barrier (Bertler *et al* 1966, Hardebo *et al* 1976) is of interest both from a general biological point of view (see Rapoport 1976) and clinically in the medical treatment of Parkinson s disease (Cotzias, Papavasiliou and Gellene 1969) It has previously been shown that the decarboxylation capacity in the wall of brain microvessels has a limit (Hardebo *et al* 1977 b) which means that the amine precursor (L DOPA) is able to penetrate into the brain parenchyma at particularly high circulating concentrations. This limit was estimated in a model where increasing doses of L DOPA were given systemically to rats, and the break through of L DOPA into the brain parenchyma via the enzymatic blood brain barrier was estimated by comparing the amount of newly formed DA in brain structures with and without decarboxylase-containing neurons (the caudate nucleus and cerebellum, respectively) The microvessels were found to efficiently trap DOPA in their walls up to a circulating level corresponding to an i.p dose of 100 mg/kg. It could be estimated that approximately 3 per cent of the exogenous L DOPA is decarboxylated at the level of the blood brain barrier whereas the remainder of the conversion takes place in peripheral tissues (Hardebo *et al* 1977 b)

In the present study the efficiency of the trapping mechanism was investigated more directly by measuring the decarboxylating capacity in the microvessel wall of the cerebral parenchyma The major problem encountered was to separate that portion of the decarboxylation taking place in the microvessels from the amine formation occurring in the monoaminergic neuron systems Two approaches were used one in which the microvascular, not neuronal, decarboxylase was inactivated by carbidopa and another in which the neurons were selectively eliminated by transection of the spinal cord leaving the microvascular amine forming capacity intact

The decarboxylase inhibitor carbidopa (α methyldopa hydrazine), is selective in the sense that it, probably due to a poor penetration through the blood brain barrier leaves neuronal decarboxylase in the central nervous system unaffected at certain dose levels which effectively inhibits peripheral DOPA decarboxylase including that in the brain microvessel wall (Bartholini and Pletscher 1969). The effect of carbidopa was checked by fluorescence microscopy Thus, incubation of brain tissue in the presence of L DOPA produced an intense fluorescence (which has been shown to be represented mainly by dopamine Bertler *et al* 1966) in the microvessel walls that on the other hand, was almost absent in tissues from carbidopa-treated animals. Persisting decarboxylase activity in the neuron system (of reserpine-pretreated animals) was indicated by an equally high neuronal fluorescence after the L DOPA incubation, whether the animals had received carbidopa or not. It was therefore assumed that most—if not all—of the decarboxylase activity measured in preparations from carbidopa treated animals reflected neuronal enzyme activity This was corroborated by the finding that the neuronal decarboxylase thus calculated comprised a much higher fraction

[illegible] decarboxylase in the caudate nucleus than in the cerebellum and spinal cord, which [illegible] a much lower number of catecholaminergic neurons. In fact, the relationship between the decarboxylase activity measured in the brain parenchyma of carbidopa-treated animals [illegible] very well to the relative concentrations of catecholamines in the spinal cord, cerebellum and caudate nucleus, respectively (see catecholamine figures compiled by Holtz[illegible] and Sharman 1972).

The monoamine neuron system in the spinal cord is entirely descending, which means that [illegible] produces an almost complete disappearance of NA caudal to the lesion (Andén *et al.* 1965). It can thus be predicted that remaining catecholamine formation occurs in the [illegible] vessel walls (and probably to a minor extent, in perivascular adrenergic nerves [illegible] to the spinal cord segmentally). After transection, the DOPA decarboxylase activity [illegible] the caudal portion of the cord dropped by 79% in the rat, which would indicate that [illegible] 70% of spinal cord DOPA decarboxylase activity resides in the vascular [illegible]. This figure agrees well with the value (79%) calculated from the carbidopa model. The corresponding figure for the intraspinal vascular DOPA decarboxylase activity in the [illegible] was lower only 45%, compared with the rat values. This is in good agreement with the lower enzyme activity that is reflected by a lower wall fluorescence in the microvessels of [illegible] under conditions equal to those for rats. The corresponding figure in rabbit experi-[illegible] presented by Andén *et al.* (1965) are somewhat lower.

In the previously mentioned *in vivo* model, in which the decarboxylating capacity in the [illegible] walls was estimated after i.p. administration of increasing doses of L-DOPA (to [illegible] pretreated with reserpine and the monoamine oxidase inhibitor nialamide), it was [illegible] that the maximum amount of DA formed in the cerebellar vessels corresponded to a [illegible] concentration of 3.12 μg/g during 20 min. The correlating value for the *in vitro* forma-[illegible] calculated from the carbidopa model (see Table I) was very close, 2.82 μg/g formed [illegible] 20 min, showing a good agreement between the two different model approaches. A [illegible] difference was found with the carbidopa model, in terms of microvascular DA [illegible], being the order caudate nucleus > spinal cord > cerebellum. The same order of [illegible] has been found when comparing the decarboxylase activity in isolated fractions of [illegible] from the caudate nucleus and cerebellum in various animals (Hardebo *et al.*, [illegible]), and it was also indicated by the cytofluorometric measurements of the formaldehyde [illegible] capillary wall fluorescence in rats given L-DOPA (Hardebo *et al.*, 1977 b).

Particularly high doses of L-DOPA have been given in the medical treatment of Parkinson's [illegible] due to the decarboxylation activity in the peripheral tissues and the efficiency of the [illegible] brain barrier to impede the passage of L-DOPA into the brain, with frequent side [illegible] as result. Based on this knowledge, including the decarboxylating capacity of the [illegible]-brain barrier (Bertler *et al.*, 1966, Bartholini *et al.* 1971), it has now been possible to [illegible] this problem: a decarboxylase inhibitor effective in peripheral tissues as well as in [illegible] capillary walls but not in the brain parenchyma, has been added to L-DOPA in the drug [illegible] of Parkinson's disease. This means that the amine precursor can now be ad-[illegible] in lower doses, reducing the incidence of side-effects.

[illegible] study was supported by the Swedish Medical Research Council (Grants No. 04X 732 and 04X 56).

calculated after vascular decarboxylase inhibition). The corresponding value in the rat was considerably lower about 45 per cent.

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From the Department of Physiology, University of Göteborg, Sweden

Effects of vasoactive intestinal polypeptide on blood flow, motility and fluid transport in the gastrointestinal tract of the cat

By

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Abstract

EKLUND, S., M. JODAL, O. LUNDGREN and A. SJÖQVIST. *Effects of vasoactive intestinal polypeptide on blood flow, motility and fluid transport in the gastrointestinal tract of the cat.* Acta physiol. scand. 1979. *105*. 461–468.

The effects of close intra-arterial infusions of vasoactive intestinal polypeptide (VIP) on gastric motility, intestinal fluid transport and colonic motility were studied in the cat. Regional blood flow was also followed in all experiments. In the stomach VIP produced a gastric relaxation and a blood flow increase. The motility response was similar to that observed when eliciting the vago-vagal reflex relaxation by distending the oesophagus. In the small intestine a hyperaemia and a decrease of net water uptake was observed. When infusing small amounts of VIP a decrease of net water uptake was seen without any change of local blood flow. Large amounts of VIP produced a transient secretory state in the small intestine. In the colon a hyperaemia was seen immediately upon starting the infusion of the drug. After 2–3 min of infusion a contraction of the colon was apparent. The administration of atropine to the animal did not significantly affect any of the responses produced by VIP. The results are discussed in relation to VIP as a possible neurotransmitter in the gastrointestinal tract.

Vasoactive intestinal polypeptide (VIP), a compound with a large spectrum of biological actions, was originally isolated from the gastrointestinal tract (Said and Mutt 1970). The molecular mass of VIP exceeds 3 000 d and it is composed of 28 amino acids. The sequence of the amino acids is fully established and it resembles glucagon and secretin. These well established hormones produce biological effects similar to VIP. It has therefore been labelled "candidate hormone" together with several other peptides recently isolated from the gastrointestinal tract (Grossman *et al.* 1974).

Immunohistochemical methods for studying the tissue localization of VIP have, however, demonstrated that most, if not all, VIP is localized to nervous tissue not only in the brain but also in peripheral organs (see e.g. Larsson *et al.* 1976). Furthermore, immunohistochemical and biochemical techniques show that VIP is predominantly localized to synaptosomes (Giachetti *et al.* 1977, Larsson 1977, Emson *et al.* 1978), suggesting that VIP may be a neurotransmitter. This proposal was further strengthened by the demonstration of VIP release from the alimentary canal upon direct electric stimulation of the vagal and

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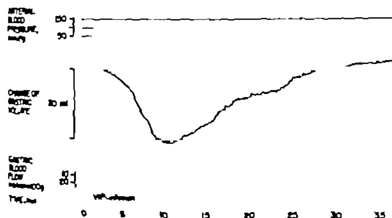
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. 1. The effects of close infusion of VIP on arterial blood pressure gastric blood flow and gastric VIP was infused at rate of 3 4 nmol/min increasing plasma concentration 0 67 pmol/l. A relaxation of the stomach reflected as downward deflection of the gastric volume recording.

3–10 cm long colonic segment for the experiment. The colonic vein was cannulated and blood was recorded continuously by drop recorder unit operating an ordinate writer as described for the Autonomic nervous influences on the colon were eliminated by cutting the pelvic nerves and the around the inferior mesenteric artery.

Colonic motility was followed by recording intraluminal volume changes with volume transducer to one end of the colonic segment, the other end being closed with ligature. Intraluminal pressure was set at 2–4 cm H_2O.

Close intra-arterial infusions of VIP were made into side-branch to the colonic artery.

and drugs

intravenously infused buffer solution contained 10 mmol $NaHCO_3$ in 100 ml 10 glucose solution was infused at rate of 0 1–0.2 ml/min.

The solution used to perfuse the lumen of the small intestine contained (mmol/l): NaCl 122, KCl 3 3 $NaHCO_3$ 21, KH_2PO_4 1 2, $MgCl_2$ $6H_2O$ 1.2, $CaCl_2$ 2.5 glucose 30. The osmolality of the solution was mOsm/kg H O

VIP was dissolved in physiologic saline at various concentrations. The infusion rates used are given in VIP was obtained from dr V Mutt at the Gastrointestinal Research Unit, Karolinska Institutet, Sweden.

(0 3–1 mg/kg b wt.) was administered during the course of the experiments.

Calculations

increase of arterial plasma VIP concentration produced by the i.a. infusion of the drug was calculated dividing the amount infused (pmol/min) by the plasma flow (ml/min) as measured when the infusion was This may represent slight overestimation of the increase of VIP concentration since blood flow increased upon infusion. However, recirculation tends to increase plasma drug concentration during infusions.

Results

Experiments on the stomach

VIP was infused close i.a. to the stomach, usually during a 3 min period, and at rates between 2 and 12 nmol/min (7–42 µg/min) which led to plasma VIP concentrations between 0 2 and 3 0 pmol/l. Changes in total gastric blood flow and gastric volume were recorded. Fig. 1 illustrates a typical experiment. Upon infusing VIP a small increase of

pelvic nerves, and also when various types of reflexes are induced via these nerves (Fahrenkrug *et al* 1978 a, b)

One of the criteria for a neurotransmitter is that it should elicit the same response in an organ when administered intravascularly as when released from nervous endings. However there is a paucity of data concerning the effects *in vivo* of intravascularly administered VIP on gastrointestinal function particularly with respect to motility. Thus, in the present study the effect of intravascularly infused VIP on motility blood flow and water absorption in the gastrointestinal canal *in situ* was investigated

Methods

Animals and general operative procedures

The experiments were performed on 14 cats, anaesthetized with chloralose (50 mg/kg b. t.) after induction with ether. The cats had been deprived of food for at least 24 h with free access to water. They exhibited no obvious signs of gastrointestinal disease.

The following procedures were common to all types of experiments performed in this study. After opening the abdomen by a midline incision, the great omentum and the spleen were extirpated. The femoral artery was cannulated after heparinization (3–5 mg/kg b.wt. i.v.) to record mean arterial blood pressure by means of a Statham pressure transducer (model P23AC). All measured parameters (see below) were recorded on a Grass polygraph. The splanchnic nerves were cut bilaterally

A slow intravenous infusion of a glucose solution containing bicarbonate (see below) was started at the time of the induction of anaesthesia and then continued throughout the experiment. This infusion had previously been shown to maintain arterial pH at a normal level, despite varying degrees of operative trauma (Haglund and Lundgren 1972)

Experiments on the stomach

A loose ligature was placed around the cardia without damaging the vagal nerve trunks. A wide-bore plastic catheter was introduced into the stomach via the pylorus through a duodenostomy and connected to a volume transducer for continuous recording of changes in gastric volume. The stomach and the volume recording system was filled with isotonic saline and arranged in such a way that the intragastric pressure could be set at and continuously adjusted to, the desired level, usually 2 cm above that at the pylorus.

Gastric blood flow was registered via a catheter in the splenic vein connected to a drop recorder operating an ordinate writer. All other veins draining the stomach were ligated. Intra-arterial infusion of VIP to the stomach was made into the hepatic artery in a retrograde direction. A vago-vagal receptive relaxation of the stomach was elicited reflexly by distension of the esophagus (Abrahamsson and Jansson 1969). This was achieved by filling a small balloon, placed in the proximal part of the esophagus, with 20 ml saline

Experiments on the small intestine

An 8–10 cm long jejunal segment was isolated with its blood supply intact. The colon and remaining parts of the small intestine were extirpated. The jejunal segment was so chosen that its arterial supply was derived from one single main branch of the superior mesenteric artery and i.a. infusions of VIP were in most experiments performed via an adjacent branch. Total venous outflow from the jejunal segment and its lymph nodes was registered via a catheter in the superior mesenteric vein, as described above.

Net intestinal transport of fluid was measured with the method described in detail by Jodal *et al.* (197 where the intestinal lumen was perfused with a body warm Krebs-glucose solution (see below) at a rate 1 ml/min in a recirculating system. Changes in motility and/or net fluid transport were registered by volume transducer connected to the system via a T tube.

Experiment on the colon

In these experiments the small intestine was extirpated and the inferior mesenteric artery was divided. This procedure has been shown not to decrease blood flow to the feline large bowel (Hultén *et al.* 19 which is amply supplied via the superior mesenteric artery. The distal part of the colon was extirpated

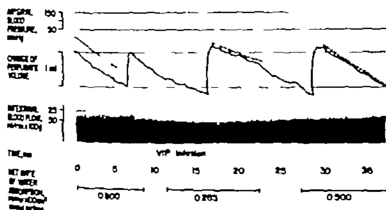


Fig. 3 The effects of close infusion of VIP on arterial blood pressure intestinal blood flow and intestinal water transport. The drug was infused at a rate of 0.017 nmol/min increasing plasma concentrations ... The upward deflections of the recording of perfusate volume were produced by adding 1 ml ... to the volume transducer

... vasodilatation it was calculated that jejunal blood flow increased 40–50 ... above ... when increasing arterial plasma VIP concentrations between 0.15 and 0.45 pmol/L. Atropine administration had no appreciable effects on the intestinal responses to VIP

Experiments on the colon

... VIP was infused i.a. to the colon while colonic blood flow and motility were ... The infusions were performed at rates varying between 1.7 and 7.1 nmol/min ..., increasing plasma VIP concentrations between 0.35 and 4.6 pmol/L. A ... expt is shown in Fig. 4. Upon starting the VIP infusion a marked blood flow ... immediately ensued, but subsided after about half a minute and levelled off at a ... flow elevation maintained throughout the infusion. After about 2 min a contraction of the colonic musculature occurred, reflected as an increased fluid volume in the volume transducer. When the VIP infusion was stopped blood flow returned rapidly to control while the colonic volume increase returned to control first 5–10 min later

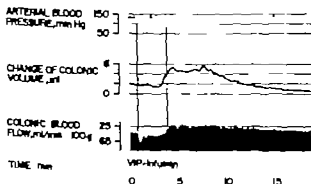


Fig. 4 The effects of close i.a. infusion of VIP on arterial blood pressure, colonic blood flow and colonic motility. VIP was infused at a rate of 3.4 nmol/min ... plasma concentration ... pmol/L. A contraction of the ... reflected as an upward deflection of the colonic volume tracing.

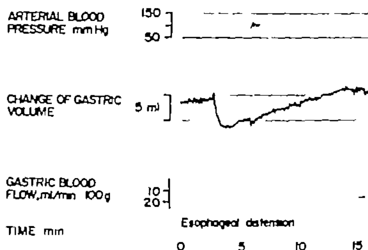


Fig. 2. The effects of distend[ing] the esophagus on arterial bl[ood] pressure, gastric blood flow [and] gastric motility. The experi[ment] was performed on the [same] animal as illustrated in Fig. 1.

gastric blood flow was observed concomitant to a pronounced increase in gastric volu[me] depicted as a downward deflection in the recording. Blood flow increased somewhat dur[ing] the period of constant VIP infusion and returned to control fairly quickly after the infusi[on]. The changes in gastric volume exhibited a characteristic, fairly fast increase during the peri[od] of drug infusion, while the return to control volume was quite slow lasting 20-30 min. [A] closely similar change of gastric volume could be elicited reflexly by distending a ballo[on] in the esophagus, as illustrated in Fig. 2 taken from the same expt. No significant change [in] gastric blood flow was observed upon esophageal distension.

In 2 expts, an attempt was made to estimate the increase of arterial plasma VIP co[n]centration necessary to produce a gastric relaxation of the same magnitude as that elicited [by] distension. It was found to be 0.3 and 0.5 μmol/l plasma.

The motility effects observed upon VIP infusions were similar in magnitude before a[nd] after giving atropine to the animal.

Experiments on the small intestine

The effects of intraarterial VIP infusions on blood flow and net water uptake in the sm[all] intestine were studied in 7 cats. VIP was infused at a rate of 0.001–7.1 nmol/min (0.003– μg/min) increasing the plasma VIP concentration 0.4–1 900 nmol/l. The high infusion ra[tes] were usually maintained for only 3 min to study the VIP effects on blood flow. At low ra[tes] infusions the experimental runs were prolonged for 10–15 min to allow recordings of V[IP] effects on net fluid transport rate in the intestine. Plasma concentrations of VIP were c[al]culated without taking into consideration recirculation of the peptide (see Methods). Fig. [3] illustrates a representative experiment, showing that blood flow is slightly increased up[on] VIP infusion while the rate of intestinal water uptake is reduced as indicated by dotted line.

The lowest increase of arterial plasma VIP concentration able to decrease intestinal n[et] fluid uptake was 2.0 nmol/l, which did not significantly increase total intestinal blood flo[w]. At high rates of infusion, increasing plasma VIP concentrations above 0.5 μmol/l, a n[et] intestinal secretion was observed continuing also after ended infusion with a slow retur[n] to control rate of absorption first about 30 min after the infusion period. With regard t[o]

[illegible] concentrations is, indeed, to be expected if VIP is a neurotransmitter in the gastro-[illegible] tract. VIP is a fairly large, water soluble compound suggesting that its capillary [illegible] is fairly low (Landis and Pappenheimer 1963). Thus VIP when released topi-[illegible] from nerve endings in the tissues, will initially show a much restricted tissue distribu-[illegible]. The compound [illegible] then be locally dispersed by diffusion and probably only to a small [illegible] reach the blood stream due to the hindrance offered by the capillary walls. Further-[illegible], potent mechanisms are likely to exist at synaptic clefts to degrade the VIP molecule [illegible] bring it back into the nerve endings. Similarly the low capillary permeability of [illegible] necessitates a high rate of intraarterial infusion of the compound to reach the same VIP [illegible] at the effector cell as that induced by the nervous release. Such principles [illegible], for instance, for the blood concentration of neurogenically released noradrenaline [illegible] intraarterially infused noradrenaline when given in concentration to match the neuro-[illegible] effects (Ljung 1970).

[illegible] of the nervous effects described above were studied with regard to their effects on [illegible] fluid transport. In the present study it was observed that one could induce [illegible] increase of net fluid absorption in the small intestine at plasma VIP concentrations well [illegible] those inducing a vasodilatation. In fact, the increase of arterial plasma VIP concentra-[illegible] that diminished net fluid uptake was only 5–10 times greater than the increase of venous [illegible] concentration observed upon mechanical stimulation of the intestinal mucosa ([illegible] et al. 1978 b). It seems therefore reasonable to conclude that VIP profoundly [illegible] intestinal net fluid transport rate at very low plasma concentrations, possibly even [illegible] such plasma concentrations seen during physiological circumstances. This conclusion is [illegible] by the findings reported from studies on isolated intestinal epithelial cells [illegible] the presence of membrane receptors with a [illegible]ery high affinity to VIP (Dupont [illegible] 1978, Laburthe et al. 1978).

[illegible] was supported by grants from the Swedish Medical Research Council (14X 2855), from the [illegible] Society for Medical Sciences, from Harald and Greta Jeanssons Fund, from Wilhelm and Martina [illegible] Fund, from Magnus Bergvalls Stiftelse and from the Faculty of Medicine, University of Göte-[illegible].

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[illegible], J. U. Haglund, M. Jodal, O. Lundgren, L. Olbe and O. B. Schaffalitzky de Mucka-[illegible]. Nervous release of vasoactive intestinal polypeptide in the gastrointestinal tract of cats, possible [illegible] implications. J. Physiol. (Lond.) In press.

To quantitate the VIP effects on colonic blood flow the increase of arterial plasma VIP concentration needed to produce as initial doubling of blood flow (Fig. 4) was determined. It varied between 1 and 4 μmol/l plasma.

Atropine had no effect on the VIP induced changes in colonic blood blow and motility. In 3 of the 5 colon expts. isoprenaline and papaverin were infused i.a. in the same way as for VIP. Vasodilatations of the same magnitude as with VIP were then produced but colonic contractions were never observed. If anything, minor relaxations occurred.

Discussion

In the present study the effects of i.a. infused VIP on various gastrointestinal functions have been investigated. In the stomach it was possible to demonstrate that VIP relaxed both the vascular smooth muscle cells and the gastric muscularis propria. Relaxatory effects of VIP on gastric smooth muscle has been reported *in vitro* (Piper, Said and Vane 1970). In contrast to the vascular effects the gastric relaxatory response to VIP under *in vivo* conditions was very longlasting returning to control first 20–40 min after ended infusion. This response closely mimics the receptive relaxation elicited by direct stimulation of the efferent vagal relaxatory fibres (Martinson 1965) or by their reflex activation by way of distending the esophagus (Abrahamsson and Jansson 1969, Fig. 2). In the small intestine VIP induced a vasodilatation and a decrease of net water transport. At high VIP concentration intestinal secretion was recorded. These observations are in agreement with earlier reports (Blood flow: Said and Mutt 1970, Thulin and Olsson 1973, Kachelhoffer *et al.* 1974. Net water transport: Berbezat and Grossman 1971, Krejs *et al.* 1978). Finally, in the colon a hyperemia and a delayed contraction was recorded upon VIP infusion. These two colonic responses are similar to those induced by pelvic nerve stimulation in animals given atropine (Hultén and Jodal 1969).

In an earlier report from this laboratory (Fahrenkrug *et al.* 1978 b) it was shown that VIP was released from the gastrointestinal tract when eliciting three different non-adrenergic, non-cholinergic neurogenic responses, *i.e.* the vagally mediated gastric receptive relaxation (Martinson 1965), the intestinal vasodilatation produced by mechanical stimulation of the intestinal mucosa (Biber *et al.* 1971) and the colonic hyperemia evoked by the pelvic nerves (Hultén *et al.* 1969). From these and other observations it was proposed that VIP is a neurotransmitter in the gastrointestinal tract. The present results strengthen this conclusion by showing that the intravascular infusion of the proposed neurotransmitter evokes physiological effects closely similar to those seen when the mentioned non-adrenergic non-cholinergic nervous mechanisms of the alimentary canal are activated.

In the study by Fahrenkrug *et al.* (1978 b) the increase of venous plasma VIP concentrations, accompanying the induction of the different nervous effects, were determined. In the present study the arterial VIP concentrations necessary for producing the same gastrointestinal effects were estimated. A comparison of the plasma VIP concentrations in the two experimental situations reveals that the nervous release of VIP into the blood stream was only 1/10 000 of the arterial VIP concentrations needed to induce quantitatively similar physiological effects upon intraarterial VIP administration. This large difference between

plasma concentrations is, indeed, to be expected if VIP is a neurotransmitter in the gastrointestinal tract. VIP is a fairly large, water soluble compound suggesting that its capillary permeability is fairly low (Landis and Pappenheimer 1963). Thus VIP when released topically from nerve endings in the tissues, will initially show a much restricted tissue distribution. The compound will then be locally dispersed by diffusion and probably only to a small extent reach the blood stream due to the hindrance offered by the capillary walls. Furthermore, potent mechanisms are likely to exist at synaptic clefts to degrade the VIP molecule and/or bring it back into the nerve endings. Similarly the low capillary permeability of VIP necessitates a high rate of intraarterial infusion of the compound to reach the same VIP concentration at the effector cell as that induced by the nervous release. Such principles hold, for instance, for the blood concentration of neurogenically released noradrenaline and intraarterially infused noradrenaline when given in concentration to match the neurogenic effects (Ljung 1970).

None of the nervous effects described above were studied with regard to their effects on transepithelial fluid transport. In the present study it was observed that one could induce a decrease of net fluid absorption in the small intestine at plasma VIP concentrations well below those inducing a vasodilatation. In fact, the increase of arterial plasma VIP concentration that diminished net fluid uptake was only 5–10 times greater than the increase of venous plasma concentration observed upon mechanical stimulation of the intestinal mucosa (Fahrenkrug *et al.* 1978 b). It seems therefore reasonable to conclude that VIP profoundly affects intestinal net fluid transport rate at very low plasma concentrations, possibly even at such plasma concentrations seen during physiological circumstances. This conclusion is corroborated by the findings reported from studies on isolated intestinal epithelial cells indicating the presence of membrane receptors with a very high affinity to VIP (Dupont *et al.* 1978, Laburthe *et al.* 1978).

This research was supported by grants from the Swedish Medical Research Council (14X 2855), from the Swedish Society for Medical Sciences, from Harald and Greta Jeanssons Fund, from Wilhelm and Martina Lundgrens Fund, from Magnus Bergvalls Stiftelse and from the Faculty of Medicine, University of Göteborg.

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Vascular anatomy and tissue osmolality in the filiform and fungiform papillae of the cat's tongue

By

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Abstract

HALLBÄCK, D.-A., M. JODAL and O. LUNDGREN. *Vascular anatomy and tissue osmolality in the filiform and fungiform papillae of the cat's tongue.* Acta physiol. scand. 1979 105 469–480.

The vascular anatomy of the filiform and fungiform papillae of the feline tongue was studied by i.a. injections of India ink. Vascular loops of various appearances were found in the types of papillae studied, *i.e.* large and the small filiform papillae and the fungiform ones. Such hairpin loops may function as countercurrent exchangers and to test this hypothesis tissue osmolality was determined in the papillae, while exposing them to various isotonic electrolyte solutions. The large filiform papillae with a vascular arrangement similar to that of intestinal villi exhibited a marked osmolar gradient from tip to base when exposed to solutions containing both glucose and sodium. If sodium and/or glucose was excluded from the solution, tissue osmolality was significantly decreased. This was also the case when the chloride ions of the solution was substituted with sulphate. The small filiform papillae are only provided with one or a few capillary loops. They exhibited a less marked osmolar gradient than the large ones and none of the different electrolyte solutions decreased the gradient. In the fungiform papillae a tissue hyperosmolality at the tip was also demonstrated. It is proposed that the papillary epithelium is provided with active transport mechanism(s) and that the papillary vessels function as countercurrent multipliers. The functional importance of these mechanisms are tentatively discussed.

The presence of a countercurrent exchanger in intestinal villi has been demonstrated (see, *e.g.* Bond *et al.* 1977, Hallbäck *et al.* 1978 a, Lundgren 1967, 1974). The anatomical basis for this mechanism are the hairpin vascular loops formed by a central artery and the surrounding densemeshed subepithelial capillary network. The main direction of blood flow in these two limbs is opposite, and since the intervascular distance between the limbs of the loop is small (about 20 μm) and the blood transit time is relatively great (4–6 s), it is possible for easily diffusible substances to diffuse from the artery to the capillaries or vice versa. If an easily diffusible compound approaches the exchanger from the luminal side, it is thus possible for this solute to diffuse from the capillaries to the artery, delaying its net blood absorption (Lundgren 1967, Svanvik 1973). Furthermore, by acting as a countercurrent multiplier, a hyperosmolar tissue region is established at the villous tip (Haljamäe *et al.* 1971, Jodal *et al.* 1978) in a way similar to that described in the renal medulla.

The papillae on the mammalian tongue seem to have a vascular arrangement resemblin that of the intestinal villi at least to judge from studies performed on the dog (for review see Hellekant 1972) Thus, a countercurrent exchanger may exist also in these papillae an the present study was initiated to test this hypothesis. Its purpose was twofold. First, it wa deemed necessary to investigate the cat with regards to its vascular anatomy in the papilla of the tongue since such studies are largely lacking. Second experiments were performec using a cryoscopic technique, to establish the possible presence of a hyperosmolar regio in the papillae, which would indicate the existence of a countercurrent exchanger

A preliminary abstract of this report has been published (Hallbäck *et al* 1978 b).

Materials and methods

A. *Experimental procedures*

The studies were performed on cats anesthetized with chloralose (50 mg/kg b.w.) after induction [illegible] The cats were heparinized and the right femoral artery was cannulated to record blood pressure by a pre sure transducer (Statham P23AC) connected to a Grass polygraph. Cats having a mean arterial blood pre sure below 90 mmHg were not included i the study

The tongue placed in a little plastic cup *in situ* was exposed to a electrolyte solution of known compos tion by dripping the solution on the dorsum of the tongue for about 30 min at a rate of 1 ml/min. I most of the experiments the sol tion was heated to a temperature of 36–38°C as controlled by a therm coupled thermistor (Electr lab, Copenhagen). In 6 expts. the tongue was quickly removed from the anim and placed in n organ bath containing a Krebs-glucose solution (see below). The bath as kept at 37 and briskly bubbled with a gas mixt re containing 4 CO_2 in oxygen.

B *Histological techniques*

T [illegible]ualize the vascular anatomy of the feline tongue the external carotid artery was cannulated in 4 expt and a mi ture (1 1) of filtered India ink and papa erine (1 mg/ml physiological saline) was injected Th injection was continued u til the tongue attained a grayish colour which requ red an injection volume (3–6 ml. After the njection the tongue was removed and the tissue was cleared by using the techniqu developed by Spalteholz (1888). After such treatment of the tissue the India ink filled blood ves were easily seen in n ord nary microscope.

In 2 expts. parts of the cat s tongue were rapidly cut out and f xed in 10 neutral formalin. The bloc were prepared for routine paraff ne embedment, cut at 4 μm, mounted and stained according to an Gieso

C. *Cryoscopic technique*

While exposed to the solution nder study the tongue was rapidly extirpated and immediately frozen isopentane precooled by liquid trogen. The tongues were then sectioned in 15 μm thick slices in a cry state at −25°C. The cutti g was performed obliquely in relation to the long axis of the tongue's papil so that one obtained crossections of papillae t various lev ls of thei lengths (see Fig. 3). The sectio were mounted on glass and examined in room at −10°C with microscope equipped with an aluminiu block, the temperature of which was kept constant by servosystem. It was increased in a step-wise fashio allowing a 4–10 min period for temperature equil bration between the metal and the tissue section. A each temperature the tissue section was photographed and the boundary between frozen and thawed tiss was estimated in relation to total papillary length. A tissue section was considered tha ed hen no i crystals were observed in the section. The tissue cryostate was callibrated using tissue sections equilibrate with solutions of varyi g osmolal ties as described in more detail by Jodal *et al* (1978).

When increasing tissue temperature step-wise, it was possible t obtain 2 5 points relating tissue osmol lity to total papillary length. These observations were treated by a computer in the following ay (Jod *et al.* 1978): to obtai the osmolality t the papillary t p line connecting the two top observations extrapolated to the 5 per cent level of total pap llary length. The upper 5 per cent were considered to composed of epithelial cells. The base value (100 per cent papillary length) was set at 312 mOsm/kg H_2O the mean value of cat plasma osmolal ty With these assumptions the computer calculated the tissue o molality at 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent of total papillary length. Moreover mean molalities in the tissue between 5 and 30 per cent nd between 5 nd 50 per cent of total papillary lengt as well as mean osmolality throughout the papillary tissue were determined.

[illegible] I. Composition of the [illegible] solutions used in this study. Concentrations are expressed in mmol/l.

	Na^+	K	Mg^{++}	Ca^{++}	Choline	Cl^-	HCO	SO^3	PO^3	Glucose	Mannitol
[illegible]-glucose	147	4.7	1.2	2.5		134	25		1.2	30	
[illegible]-mannitol	147	4.7	1.2	2.5		134	25		1.2		30
[illegible]-glucose		4.7	1.2	2.5	147	134	25		1.2	30	
[illegible]-mannitol		4.7	1.2	2.5	147	134	25		1.2		30
[illegible]-glucose-[illegible]	147	4.7	1.2	2.5			30	62	1.2	30	61

) *Solutions*

The physiological saline solution contained 0.9 g NaCl per liter water. Its osmolality measured 292 mmol/kg H_2O. The composition of the other electrolyte solutions used are given in Table I.

Statistics

Statistical significance was tested using Wilcoxon nonparametric tests (Siegel 1956).

Results

Histological observations

In the cat's tongue 3 different types of papillae were observed, *i.e.* filiform, fungiform and [illegible]vallate. The present study was primarily devoted to the study of the vascular anatomy of the filiform papillae, and to some extent also to the fungiform ones.

1. *Filiform papillae.* The filiform papillae were of different size and shape, and it was possible to differentiate between two main types in the cat according to the arrangement of the vessels, location on the tongue and extent of keratinization. *I.* A small type with one or two vascular loops (Fig. 1). The intervascular distance measured about 50 µm, as estimated

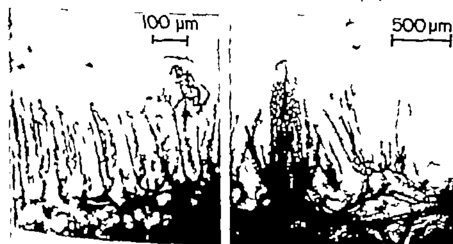


Fig. 1 Fig. 2

Fig. 1 The vasculature of papillae on the cat's tongue as demonstrated by an i.a. injection of India ink. In the right part of the Fig. a fungiform papilla is shown, while the rest of the Fig. illustrates the appearance of small U-form papillae. For further details, see text.
Fig. 2 The vascular architecture of large filiform papillae (left) and fungiform papillae (right). For details, see text.

The papillae on the mammalian tongue seem to have a vascular arrangement resembling that of the intestinal villi at least to judge from studies performed on the dog (for review see Hellekant 1972). Thus, a countercurrent exchanger may exist also in these papillae and the present study was initiated to test this hypothesis. Its purpose was twofold. First, it was deemed necessary to investigate the cat with regards to its vascular anatomy in the papillae of the tongue, since such studies are largely lacking. Second, experiments were performed using a cryoscopic technique, to establish the possible presence of a hyperosmolar region in the papillae, which would indicate the existence of a countercurrent exchanger.

A preliminary abstract of this report has been published (Hallbäck *et al.* 1978 b).

Materials and methods

A Experimental procedures

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The tongue, placed in a little plastic cup *in situ* was exposed to an electrolyte solution of known composition by dripping the solution on the dorsum of the tongue for about 30 min at a rate of 1 2 ml/min. In most of the experiments the solution was heated to a temperature of 36–38°C as controlled by a thermocoupled thermistor (Electrolab, Copenhagen). In 6 expts. the tongue was quickly removed from the animal and placed in an organ bath containing a Krebs-glucose solution (see below). The bath was kept at 37 and briskly bubbled with a gas mixture containing 4 CO_2 in oxygen.

B Histological techniques

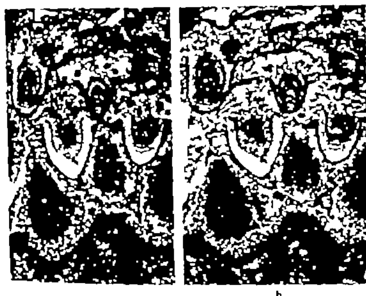
To visualize the vascular anatomy of the feline tongue the external carotid artery was cannulated in 4 expts and a mixture (1 1) of filtered India ink and papaverine (1 mg/ml physiological saline) was injected. The injection was continued until the tongue attained a grayish colour which required an injection volume of 3–6 ml. After the injection the tongue was removed and the tissue was cleared by using the technique developed by Spalteholz (1888). After such treatment of the tissue, the India ink filled blood vessels were easily seen in an ordinary microscope.

In 2 expts. parts of the cat's tongue were rapidly cut out and fixed in 10 % neutral formalin. The blocks were prepared for routine paraffine embedment, cut at 4 μm, mounted and stained according to van Gieson.

C. Cryoscopic technique

While exposed to the solution under study the tongue was rapidly extirpated and immediately frozen in isopentane precooled by liquid nitrogen. The tongues were then sectioned in 15 μm thick slices in a cryostate at −25°C. The cutting was performed obliquely in relation to the long axis of the tongue's papillae so that one obtained crossections of papillae at various levels of their lengths (see Fig. 3). The sections were mounted on glass and examined in a room at −10°C with a microscope equipped with an aluminum block, the temperature of which was kept constant by a servosystem. It was increased in a step-wise fashion allowing a 4 10 min period for temperature equilibration between the metal and the tissue section. At each temperature the tissue section was photographed and the boundary between frozen and thawed tissue was estimated in relation to total papillary length. A tissue section was considered thawed when no crystals were observed in the section. The tissue cryostate was calibrated using tissue sections equilibrated with solutions of varying osmolalities as described more detail by Jodal *et al.* (1978).

When increasing tissue temperature step-wise, it was possible to obtain 5 points relating tissue osmolality to total papillary length. These observations were treated by computer in the following way (Jodal *et al.* 1978) to obtain the osmolality at the papillary tip line connecting the two top observations extrapolated to the 5 per cent level of total papillary length. The upper 5 per cent were considered to be composed of epithelial cells. The base value (100 per cent papillary length) was set at 312 mOsm/kg H_2O, the mean value of cat plasma osmolality. With these assumptions the computer calculated the tissue osmolality at 5 10, 20, 30, 40, 50, 60, 70 80 and 90 per cent of total papillary length. Moreover mean osmolalities in the tissue between 5 and 30 per cent and between 5 and 50 per cent of total papillary length as well as mean osmolality throughout the papillary tissue were determ



b

Fig. 3. *Left panel* Histological section of the tongue of cat photographed through microscope at temperature corresponding to freezing point depression of solution with an osmolality of 340 mOsm/kg H_2O. The tissue is sectioned so that cross sections of large filiform papillae are obtained. The tissue sections (arrows) close to the lumen (L, upper part of Fig.) are completely thawed. In the deeper part of the tissue (lower part of Fig.) ice crystals are seen. *Right panel* The same histological section as in the left panel photographed at temperature corresponding to freezing point depression of solution with an osmolality of about 315 mOsm/kg H_2O. At this temperature the whole tissue is melted.

thawed. Hence, one may conclude that a gradient of tissue hyperosmolality exists, the highest osmolality being found at tips of the papillae. When the temperature of the tissue was increased to one corresponding to an osmolality of 310 mOsm/kg H_2O or less, the whole tissue including the solution on its surface, thawed.

Fig. 4 summarizes the results of 6 expts. in which the tongue has been exposed to isotonic solutions with varying amounts of glucose and sodium. The left panel illustrates the results on the large filiform papillae and the right panel the results on the small ones. Exposing the papillae to a Krebs-glucose solution produced a marked osmolar gradient along the length of the large papillae, the tip osmolality being on an average above 800 mOsm/kg H_2O (Table II). A similar gradient was also found in the small papillae although less pronounced.

Substituting all sodium ions in the solution with choline (choline-glucose solution) markedly decreased tissue osmolality in the large papillae while no significant effect was seen in the small filiform papillae (Fig. 4). Similarly removing glucose from the Krebs-glucose solution decreased tissue osmolality in the large papillae but not in the small ones (Fig. 5). Excluding glucose from the sodium free solution, on the other hand, produced no significant effect on tissue osmolality in any type of filiform papillae (compare Fig. 4 and 5).

In 6 expts. the chloride ions of the Krebs-glucose solution was substituted with sulphate ions keeping osmolality constant by addition of mannitol. This experimental procedure markedly depressed tissue osmolality in the large papillae, while only a small, insignificant effect on the osmolality was observed in the small ones (Fig. 6).

TABLE II Tip and mean tissue osmolality in mOsm/kg H O in various portions of large filiform papil of the cat s tongue during the different experimental conditions of this study Mean alues + S1

	Tip	From 5 to 30 of illous length	From 5 to 50 of villous length	Whole villous
Krebs-glucose (n = 12)	833 ± 53	729 ± 79	662 ± 19	545 ± 1
Krebs mannitol (n = 10)	594 ± 3	528 ± 15	489 ± 11	464 ± 11
Choline-glucose (n = 19)	414 ± 13	391 ± 10	374 ± 9	347 + 6
Choline-mannitol (n = 12)	475 ± 20	430 ± 13	400 ± 9	36_ ± 7
Krebs-glucose-sulphate (n = 16)	361 ± 15	343 ± 7	337 ± 6	326 ± 3
Ischemia (n = 18)	414 ± 11	395 ± 9	377 ± 7	351 + 5
N Cl 38°C (n = 4)	639 ± 24	571 ± _5	528 ± _3	456 ± 18
NaCl 20–22 C (n = 12)	448 ± 57	387 ± 76	373 ± 20	352 ± 12

on the photographs taken of the histological specimens. These papillae were situated on th tip and lateral sides of the tongue. The keratinization was scanty or none. 2 A larger co or tusk-shaped type with a central vessel presumably an artery and a well developed cap lary network (Fig. 2) This type was situated on the dorsum of the tongue, and the papill were partly covered with a hornpickle. The distance between the central vessel and th capillary network measured about 100 μm. Within each of the two groups more simple an more complicated types occurred.

2. *Fungiform papillae* The fungiform papillae also differed in shape and vascular arrang ment and two main types were discerned *1* In the fungiform papillae on the tip and th sides of the tongue, the vascular supply consisted of a single vessel probably an arterial on This vessel formed in the upper half of the papillae a few capillary loops (Fig. 1). *2* On th dorsum of the tongue the fungiform papillae were more heterogeneous in shape and vascul arrangement In one type a ring of capillary loops were formed in the upper 2/3 of the papi lae, the arterial and venous supply consisting of several vessels (Fig. 2) Other papillae we provided with a capillary network all the way from tip to base and a centrally runni arterial vessel.

B Cryoscopic findings

1 *Filiform papillae* The results of the cryoscopic investigations on the filiform papill are given in Fig. 3–9 (osmolality gradients along the papillae) and in Tables II and I (osmolality in various parts of the papillae) The observations on the filiform papillae a described separately for small (type 1 as described above) and for the large papillae (type 2

After exposing the dorsum of the cat tongue for 30 min to a Krebs-glucose solution, was possible to demonstrate the presence of a hyperosmolar region at the tips of both th filiform and fungiform papillae. A representative experiment on the large filiform papill is illustrated in Fig. 3 The left part of this figure shows the appearance of the frozen tiss sections at a temperature corresponding to 540 mOsm/kg H_2O It can be seen that som cross sections of papillae in the upper part of the figure, *i.e* in the tip portion of the papi lae, have thawed. In the lower part of the figure black "grains" *i.e* ice crystals, are abundan The right part of the figure illustrates the appearance of the tongue papillae at a temperatur corresponding to an osmolality of about 315 mOsm/kg H_2O In this figure the whole tissu



b

Fig. 3 *Left panel* Histological section of the tongue of cat photographed through microscope at a temperature corresponding to freezing point depression of a solution with an osmolality of 540 mOsm/kg H_2O. The tissue was sectioned so that cross sections of large filiform papillae are obtained. The cross sections (arrows) close to the lumen (L, upper part of Fig.) are completely thawed. In the deeper part of the tissue (lower part of Fig.) ice crystals are seen. *Right panel* The same histological section as in the left panel photographed at temperature corresponding to freezing point depression of solution with an osmolality of about 315 mOsm/kg H_2O. At this temperature the whole tissue is melted.

thawed. Hence, one may conclude that a gradient of tissue hyperosmolality exists, the highest osmolality being found at tips of the papillae. When the temperature of the tissue was increased to one corresponding to an osmolality of 310 mOsm/kg H_2O or less, the whole tissue including the solution on its surface, thawed.

Fig. 4 summarizes the results of 6 expts. in which the tongue has been exposed to isotonic solutions with varying amounts of glucose and sodium. The left panel illustrates the results on the large filiform papillae and the right panel the results on the small ones. Exposing the papillae to a Krebs-glucose solution produced a marked osmolar gradient along the length of the large papillae, the tip osmolality being on an average above 800 mOsm/kg H_2O (Table II). A similar gradient was also found in the small papillae although less pronounced. Substituting all sodium ions in the solution with choline (choline-glucose solution) markedly decreased tissue osmolality in the large papillae while no significant effect was seen in the small filiform papillae (Fig. 4). Similarly removing glucose from the Krebs-glucose solution decreased tissue osmolality in the large papillae but not in the small ones (Fig. 5). Excluding glucose from the sodium free solution, on the other hand, produced no significant effect on tissue osmolality in any type of filiform papillae (compare Fig. 4 and 5).

In 6 expts. the chloride ions of the Krebs-glucose solution was substituted with sulphate and keeping osmolality constant by addition of mannitol. This experimental procedure markedly depressed tissue osmolality in the large papillae, while only a small, insignificant effect on the osmolality was observed in the small ones (Fig. 6).

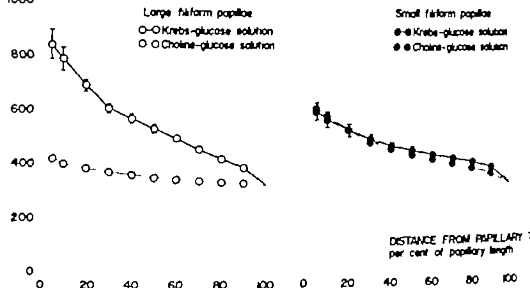


Fig. 4 Tissue osmolal ty large and small tongue papillae when exposed to isotonic Krebs-glucose choline glucose solutions. The temperature of the solutions was maintained at 36–38°C. Bars denote S At several po nts of the Fig. the S.E. is so small that it f lls within the area of the f lled circle. For numb of observations, see Tables II and III

Exposing the tongue to water markedly decreased osmolality in the small filiform pap lae (Fig. 7), a decreasing osmolality from base to tip being observed. In the large papill on the other hand, no such marked hypoosmolality was apparent. In fact it was sometim difficult to detect any decrease below isotonicity since the whole papilla thawed at a tempe ture just below the isotonic one The general impression from these experiments was th exposing the large papillae to water decreased tissue osmolality to about 250 mOsm/ H_2O at most No clearcut gradient of the type illustrated on Fig. 7 could, however demonstrated

In one series of expts. the tongue was exposed to an isotonic saline solution at two d ferent temperatures. Fig. 8 illustrates the results obtained in the large papillae, which we

TABLE III Tip and mean tissue osmolality in mOsm/kg H_2O in various portions of small filiform papil of the cat tongue dur g the different experimental conditions of this study Mean values ± S

	Tip	From 5 t 30% of villous length	From 5 to 50 of villous length	Whole villous
Krebs-glucose (n = 15)	589 ± 30	528 ± 22	497 ± 17	476 ± 13
Krebs-man ltol (n = 15)	568 ± 21	528 ± 17	502 ± 15	490 ± 11
Choline-glucose (n = 18)	601 ± 13	544 ± 9	501 ± 7	434 ± 6
Choline-mannitol (n = 13)	623 ± 22	567 ± 17	5?9 ± 16	497 ± 15
Krebs-glucose-sulphate (= 17)	528 ± 20	480 ± 17	450 ± 14	399 _ 9
Ischemia (n = 18)	480 ± 8	452 ± 6	431 ± 5	390 ± 5
Water (n = 18)	116 ± 6	170 ± 7	195 ± 6	241 ± 5

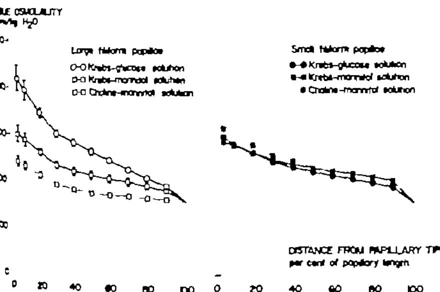


Fig. 5. Tissue osmolality in large and small filiform papillae of the cat tongue recorded when exposed to Krebs-glucose, Krebs-mannitol or choline-mannitol solutions. For further details of the figure, consult the legend to Fig. 4.

the only ones studied in this series of expts. The lowering of temperature decreased tissue osmolality significantly.

Finally in 6 expts. the tongue was removed and placed in a well oxygenated organ bath containing Krebs-glucose. The results obtained are illustrated in Fig. 9 (ischemia). A significantly decreased tissue osmolality was apparent in both types of papillae although the depression was most marked in the large ones.

2. *Fungiform papillae.* Due to their shape and limited number it was not possible to cut the fungiform papillae in cross sections in the way shown in Fig. 3 for the filiform ones. For such reasons it was difficult to determine the precise localisation of an observed cross-section along the length of a papilla. However it was quite clear that a hyperosmolar region was also present in the fungiform papilla. In the series of expts. with a Krebs-glucose solution, an attempt was made to estimate the tip osmolality. It was shown to be at least 500 mOsm/kg H_2O in the small type of fungiform papillae and at least 600 mOsm/kg H_2O in the large ones.

Discussion

In this study both anatomical and physiological observations have been reported, suggesting the presence of a countercurrent exchanger in the filiform and fungiform papillae of the cat tongue. In an anatomical investigation it was demonstrated that hairpin vascular loops are present in both types of papillae. The appearance of the vascular architecture observed in this study was the same as that earlier described for the papillae in dog and man (Hellekant

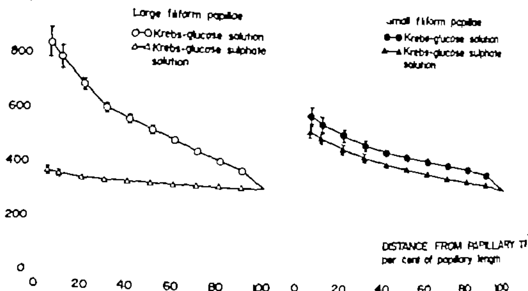


Fig. 6. Tissue osmolality in large and small filiform papillae when exposed to Krebs-glucose or Krebs-glucose-sulphate solutions, illustrated in the same way as in Fig. 4

1972). The intervascular distance between the two limbs of the vascular loops could be measured in the filiform papillae In the small type it was estimated to 50 μm and in the large type to about 100 μm A substance with a free diffusion coefficient in water of 10^{-5} cm²/s would reach a 50 % diffusion equilibrium across the mentioned distances in 0.1-0.2 and about 0.5 s, respectively The diffusion process is, hence, fairly rapid across the measured intervascular distance However the possible functional effects of the vascular arrangement in the papillae do not solely depend on time for diffusion but also on such hemodynamic parameters as e.g. mean transit time in the vascular loops. No data are at present available on the detailed hemodynamics of the papillae of the tongue.

The possible functional implications of the vascular arrangement was investigated by determining tissue osmolality with a cryoscopic technique originally developed for the small intestine (Jodal *et al* 1978). The tongue was exposed to different electrolyte solutions and marked hyperosmolality was observed at the papillary tip when exposing the tongue to solutions containing both sodium and glucose, two molecules known to interact and facilitate their mutual transepithelial transport (Crane 1968) An osmolality gradient was also observed toward the base of the papillae Furthermore, performing experiments in the absence of blood flow markedly decreased the measured tissue osmolality particularly in the large filiform papillae (Fig. 9)

These observations are fully consistent with the presence of a countercurrent multiplier in the papillae of the tongue. Fig. 10 depicts the proposed mechanisms in the large and small filiform papillae. The active transport of a solute increases its plasma concentration in the subepithelial capillary network at each point along the length of the papillae. In the large papilla such an active transport would produce a higher concentration of the solute in the

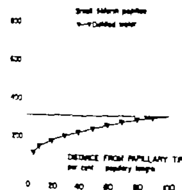


Fig. 7

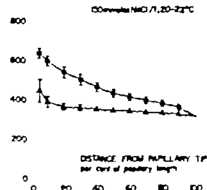


Fig. 8

Fig. 7. Tissue osmolality in the small filiform papillae when the tongue was exposed to distilled water. Presented in the same way as in Fig. 4.

Fig. 8. Tissue osmolality in the large filiform papillae when the tongue was flushed with an isotonic sodium chloride solution at two different temperatures. For further details of the figure, consult legend to Fig. 4.

capillaries than in the central arterial vessel and the solute may diffuse along this concentration gradient to be brought back towards the tip by the central vessel. If the solute capillary permeability is low water may move in the opposite direction driven by the osmotic forces created by the active transport mechanism. In the small filiform papillae with only one or a few capillary loops the concentration of the actively transported solute is higher in the draining limb of the loop than in the supplying one (right panel of Fig. 10). As in the large filiform papilla the solute may move along its concentration difference and/or water may be transferred secondary to osmotic forces.

The described mechanisms produce the same end result, *i.e.* an increasing tissue hyperosmolality from base to tip in the papillae as reported in this study. The results clearly suggest that the countercurrent multiplier is most efficient in the large filiform papillae as compared to the small ones. This difference may be ascribed to differences in the solute transport capacity or to the obvious differences in vascular anatomy (Fig. 1 and 2).

It might by argued that the demonstration of a hyperosmolar region in the papillary tip and a decreasing osmolar gradient along the papilla may be attributable to the presence of solute pumps with different transport capacities. If so blood flow would tend to decrease the gradient by carrying away some of the absorbed solute, and one would find a higher tissue osmolality during *in vitro* conditions than *in vivo*. As shown in Fig. 9 the tissue osmolality was markedly decreased *in vitro* particularly in the large papillae which argues against the view that the observed hyperosmolar compartment was solely due to the distribution of solute pumps.

An attempt was made in this study to elucidate which solute that established the tissue hyperosmolality. For such reasons the compositions of the solutions were varied particu-

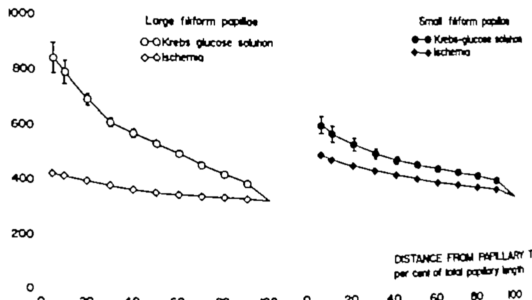


Fig. 9 Tissue osmolality in large and small filiform papillae when exposing the tongue to a Krebs-gluc solution *in* *itro* (circles) or *in* *itro* (squares). For further details of the figure, consult legend to Fig 4

larly with respect to their contents of sodium, chloride and glucose. In the large filifo papillae the exchange of sodium with choline or glucose with mannitol markedly decreas the observed papillary hyperosmolality (Fig. 4 and 5). These observations suggest that active transport of sodium is of prime importance for establishing the hyperosmolality the same way as has been shown in the cat intestine (Haljamäe *et al* 1973 Jodal *et al* 197 Hence, the epithelium on the large filiform papillae, seems to be provided with polariz cells that seem to be capable of moving sodium against a concentration gradient.

Exposing the large papillae to a solution in which the chloride ions had been substitut with sulphate ions also markedly lowered the tissue osmolality as compared to the one se during exposure to a Krebs-glucose solution Sulphate ions, like most divalent ions, poorly transported across most epithelia (Wilson 1962) probably including also the epit

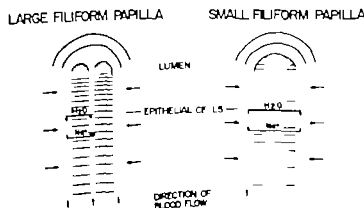


Fig. 10. The proposed count current multiplication mech isms in large and small filifo papillae schematically illustrat The increased osmolality in tissue is indicated by an creased number of horizon lines per unit length. For furt details of the figure, see text.

lum of the tongue. If so, the inability of the sulphate ions to move across the epithelium can secondarily lead to a low transport rate of sodium explaining the observed low tissue osmolality. Alternatively all the results in this study obtained with solutions of varying composition may be explained by the presence of an active chloride transport mechanism.

It might be argued that the presence of hornpickle on the large filiform papillae would provide such a hindrance to solute transport that e.g. glucose and sodium would not reach the epithelium. However the hornpickle only covers part of the papilla. Furthermore, the tongue epithelium of the rat has been shown to have a measurable permeability to glucose (Nattrass 1971). Finally the frog skin with a histological appearance similar to that of the mammalian tongue is provided with active transport systems.

The results obtained on the small filiform papillae differed markedly from that described above for the large ones. The osmolar gradient found in the small papillae was largely unaffected by variations in sodium and glucose contents of the applied solutions (Fig. 4 and 5). These observations suggest that no active transport of sodium was involved in the establishment of the tissue hyperosmolality. The Krebs-glucose solution containing sulphate did not significantly decrease the gradient. This observation seems to suggest that the chloride ion is not involved in producing the tissue osmolality and that other transport mechanisms must be responsible. Furthermore, the water permeability of the small papillae was greater than that of the large ones to judge of the marked hypotonicity found in the small but not in the large papillae (Fig. 7).

The analysis of the fungiform papillae could for technical reason not be carried out in the detailed way that is described for the filiform papillae above. This was disappointing since the fungiform type, provided with taste buds, seems from a functional point of view more interesting than the filiform papillae. It was, however clearly demonstrated that a hyperosmolar compartment also existed in the tissue of these papillae.

The physiological significance of the proposed countercurrent multiplier in the tongue is not known. With the hyperosmolality present in the papillae it seems probable that the tongue absorbs water along the transepithelial osmotic gradient. The forces involved may be very great since 1 mosm correspond to a pressure of about 20 mmHg. Assuming reflection coefficient of 1 the transepithelial osmolality difference across the papillary tip (around 500 mOsm/kg H_2O) corresponds to a pressure difference of 10 000 mmHg. Although such forces should produce a fast absorption of water it must be quantitatively of minor importance compared to the absorptive capacity of the small bowel due to the difference in absorptive area.

The fungiform papillae are supplied with taste buds and the countercurrent exchange may hence, possibly be important for taste. One may only speculate on this and two tentative proposals will be made. First, it has been demonstrated in the small intestine that its exchanger delays the net blood absorption of lipophilic solutes due to their trapping in the exchanger (Svanvik 1973 Jodal *et al* 1977). A comparatively high concentration of the lipid soluble compound is then built up in the tissue due to its "recirculation" in the villus. In a similar way one may envisage a trapping of lipid soluble "taste" compounds in the exchanger delaying their net blood transport. Such a mechanism would maintain a high tissue concentration of the compound for longer time than in the absence of an exchanger

Second, it may be inferred from the results of this study that the extracellular concentra tion of electrolytes, including potassium, may be much higher than normal at the papillar tips. Such high electrolyte concentrations may be of importance for the activation of th taste buds and possibly also of the free nerve endings in the filiform papillae.

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Plasma catecholamines, cyclic AMP and metabolic substrates in hemorrhagic shock of the rat. The effect of adrenal demedullation and 6-OH-dopamine treatment

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Abstract

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Plasma catecholamines, cyclic AMP and metabolic substrates in hemorrhagic shock of rats was studied in 4 groups of animals: 1) Control rats, 2) rats with adrenal demedullation, 3) rats with 6-OH-dopamine induced chemical sympathectomy and 4) rats with combined demedullation and sympathectomy. The rats were bled to a systemic blood pressure of 35 mmHg.

The basal plasma level of noradrenaline (NA), adrenaline (A) and dopamine (DA) in control animals was each about 1 nmol/l. After hemorrhage for 1 h the A levels had reached 50 nmol/l and there was little further rise after 4 h. This rise was eliminated by demedullation but unaffected by sympathectomy. NA levels rose continuously in the control and in the sympathectomized rats. At 1 h the level was about 4 nmol/l and at 4 h about 20 nmol/l. The demedullated rats showed a 3-fold increase in circulating NA, while little or no change was seen in the combined demedullated and sympathectomized rats. DA levels did not change in any of the groups during the first hour, but were markedly elevated after 4 h of hypotension.

Cyclic AMP and glucose levels in plasma showed a rapid increase 1 h after hemorrhage and thereafter returned to or below basal values. Demedullation largely prevented the increase, while sympathectomy had no effect. The increase in lactate and pyruvate levels were diminished but not eliminated by either sympathectomy or demedullation. Glycerol levels were unchanged and FFA decreased in all groups of rats.

The results show that the adrenal medulla is the dominating source of plasma catecholamines in hemorrhagic shock in rats. The initial increase in plasma glucose and cyclic AMP appear to be largely mediated by adrenal A. The subsequent fall in these parameters and derangement of circulatory homeostasis are not, in the present shock model, primarily due to a failure of catecholamine secretion, but rather to a decreased responsiveness of peripheral tissues to catecholamine stimulation.

It is well known that the sympatho-adrenal system is activated by hemorrhage (see Chien 1967). Thus, there are several reports that in dogs bled to 40 mmHg plasma adrenaline (A) levels increase 50-fold and plasma noradrenaline (NA) levels about 10-fold (see Watts 1965). There is evidence that a major source of plasma catecholamines in this condition is the

adrenal medulla (Watts 1965) Recent advances in the methodology of catecholamine assay in plasma have, however indicated that most, if not all the older determinations are in error Moreover most of the data on catecholamine levels in shock are obtained in larger animal such as cat and dog, while much of the data concerning survival etc. are obtained on sma rodents such as the rat

We have studied the effect of adrenal demedullation and/or chemical sympathectomy on the metabolic responses to bleeding in a previously characterized modified Wiggers shock model in the rat (Whigham and Weil 1966 Farnebo and Hamberger 1977 Farneb *et al* 1977) A new sensitive and specific method for the determination of plasma A, N and dopamine (DA) levels was used (Keller *et al* 1976 Mefford *et al* 1977 Hallman *et a* 1978) to study the induced changes in plasma catecholamine levels.

Several metabolic alterations result from hemorrhage. There is an increase in hepati glycogenolysis and glucose output (see Levenson *et al* 1961), which leads to elevated plasm glucose levels (see Järhult 1975) This response is most commonly attributed to A release from the adrenals (*e g* Engel 1943 Halmagyi *et al* 1967 Hiebert *et al* 1973), but Järhu (1975) suggested that activation of sympathetic nerves to the liver was at least as important mechanism Plasma cyclic AMP also increases during bleeding in dog (McArdle *et al* 197 and rat (Farnebo *et al* 1977) It is not known to what extent sympathetic nerves and th adrenal medulla contribute to the elevation of plasma cyclic AMP following hemorrhage Bleeding also induces several other metabolic alterations such as increased lactate an pyruvate levels as well as changes in fat metabolism (*e g* Weil and Afifi 1970 Kovach *et a* 1970 Farnebo *et al* 1977) The role of circulating and/or locally released catecholamine in the induction of these metabolic events is similarly unknown. A second aim of the presen study has therefore been to study the metabolic changes induced by hemorrhage simul taneously with the determination of catecholamines.

Material and Methods

M le Sprague-Dawley rats weigh ng about 250 g were used They were given water d libitum and fed standard diet which was withheld bout 18 h before the experiment. Four experimental groups of ra were used. 1 *U treated controls.* 2. *Demedullated rat* About 3 weeks before the hemorrhage experiment the rats were anesthetized w th sod um pentobarbital 60 mg/kg i.m. A midline skin incision was made the back through which both adrenals could be removed. The cortex and medulla were separated under microscope and numerous small pieces of the cortex tra splanted under the renal capsule (Basso *et* 1970) The rats survived f r 3 weeks postoperatively and their weight gain on the standard diet as th same as that of the controls. Since animals with deficiency drenal cortical function rapidly lose weight and die with weeks (Hahn 1969) this suggests that the adrenal cortical transplants were functioning When the rats were killed, it was found that much of the cortical material had been revascularized in agree ment with the results from the original description of the transplantation method (Basso *et al.* 1970), which paper also results of corticosteroid assays re given. Numerous smears of the transplants were mad to try to localize ay remna ts of the medulla usi g fluorescence histochemistry but no such tissue could be seen. 3 *Chemically sympathectomized rat* The rats were injected with 6-OH DA 50 mg/kg i.v 3 an 2 days before the hemorrhage experiment. When these rats were k lled whole mounts of iris were examined in the fluorescence microscope to control the degree of sympathectomy Only one to three terminal system could be seen in each iris which demonstrates h gh degree of denervation after 6-OH DA at least in thi tissue. 4. *Demedullated and sympathectomized rat* Both the procedures described under 2 nd 3 were per formed.

Experimental procedure

The experimental haemorrhage model used is principally that described by Whigham and Weil (1966) with minor modifications (Farnebo and Hamberger 1977). The rats were anaesthetized with sodium pentobarbital 60 mg/kg ip. Heparinized catheters were inserted in both femoral arteries and heparin 300 U/kg iv was injected. The right catheter was connected to a Statham transducer and the blood pressure continuously recorded on a Grass polygraph. After a rest period of 30 min the left femoral catheter was connected to a plastic heparinized syringe connected to a mercury manometer which was adjusted to 35 mmHg. Blood was then allowed to flow out into the reservoir. The blood pressure, pulse rate and shed blood volume were continuously recorded. After 15 min the blood pressure was 40–45 mmHg in all groups of rats, and after 45 min the arterial blood pressure had reached 35 mmHg. Samples of 2 ml blood were taken for analysis of catecholamines and metabolites by disconnecting the femoral catheter to the reservoir and allowing the blood to drip into ice chilled tubes. In the rats subjected to haemorrhage samples were taken at the beginning of bleeding and after the scheduled time of bleeding. In the non-bled controls, blood was taken only once.

Determination of catecholamines

After centrifugation of the blood, plasma samples of 1 ml were taken for analysis. After addition of dihydroxybenzylamine as internal standard and addition of sodium metabisulphite to prevent oxidation the catecholamines were adsorbed on 15 mg of alumina (BDH, Poole, England). After washing, the catecholamines were eluted with 50 µl PCA, 20 µl of which was injected into a high performance liquid chromatograph equipped with an electrochemical detector. This method has been developed by Adams and co-workers (Keller et al. 1976, Mefford et al. 1977) and applied to plasma (Hallman et al. 1978). For detailed description and discussion of the method the reader is referred to the latter paper.

Determination of cyclic AMP

Cyclic AMP was generally determined directly on unpurified plasma samples collected in EDTA (final concentration 10 mM) essentially as described by Brown et al. (1972). Plasma samples of 50 µl were incubated 2 h at 4 °C in 290 µl (or 10 µl in 290 µl) 90 mM Tris-Cl and 7 mM β-mercaptoethanol, 5 mM EDTA and 0.1 M NaCl, containing ^3H-cyclic AMP (1 pmol) and sufficient amount of binding protein to ensure 30–35% binding of the labelled cyclic AMP. Purification of the plasma cyclic AMP by perchloric acid deproteinization followed by anion or cation (AG 1 × 8 or AG 50 × 8) exchange chromatography gave identical values to unpurified samples when corrections for losses were made. Treatment with phosphodiesterase (partially purified from rat brain) removed 90–95% of the apparent cyclic AMP activity. Serial dilutions of the plasma fell on the standard curve. The authenticity of the cyclic AMP was therefore considered established.

Determination of metabolic substrates

Glucose was measured by a glucose oxidase method (Glox), glycerol enzymatically (Laurell and Tibbling 1966), FFA colorimetrically (Laurell and Tibbling 1967), on plasma. Lactate and pyruvate in samples of blood precipitated with 0.4 M perchloric acid was determined enzymatically (TCR, TCC, Boehringer).

Separation of cyclic AMP from other nucleotides and bases

In the experiments where the fate of ^3H-cyclic AMP was studied, plasma and tissues (liver, kidney) were deproteinized by homogenization in perchloric acid (final concentration 0.4 M). After standing in the cold for 30 min the samples were centrifuged and the supernatant neutralized with 4 M KOH and 1 M Tris-base. An aliquot was treated with equal volumes of 0.4 M $ZnSO_4$ and 0.4 M Na_2CO_3, which precipitates all labelled nucleotides except cyclic AMP (Chen and Lin 1974). The radioactivity in the precipitate and supernatant was then counted by liquid scintillation. An aliquot of this supernatant or of the original neutralized supernatant was chromatographed on short (0.7 × 1.5 cm) AG 1 × 8 (200–400 mesh, Cl) columns. Nucleosides were eluted by 0.1 M Tris-Cl pH 7.4 and nucleotides by 2 M HCl. The radioactivity was thus separated into fractions: cyclic AMP, other nucleotides, nucleosides and bases.

Drugs

^3H-Adenosine 3',5'-cyclic phosphate as the ammonium salt (26 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England, unlabelled cyclic AMP, AMP, ADP, ATP and adenosine from Boehringer, Mannheim, FRG. The cyclic AMP binding protein was prepared as described by Brown et al.

TABLE I The effect of demedullation (demed) chemical sympathectomy (6-OH DA) and of the combin treatment on arterial blood pressure and on arterial catecholamine levels. Results are given means ± S.E. Number of observations is given within parenthesis.

Treatment		BP_H (mmHG)	A (nM)	NA (nM)	DA (nM)
Control	0 h	106±4 (20)	0.92±0.23 (12)	1.16±0.14 (12)	1.08±0.36 (12)
	4 h		3.47±1.42 (3)	2.07±0.31 (3)	2.77±0.84 (3)
Demed	0 h	97±3 (20)	ND[a] (11)	0.70±0.07 (11)	TA[b] (11)
6-OH DA	0 h	93±3 (18)	0.71±0.21 (10)	0.68±0.46 (10)	TA (10)
Demed + 6-OH DA	0 h	90±2 (2_)	TA[b] (10)	0.4 ±0.07 (10)	TA[b] (10)

Not detectable.
[b] Trace amounts found in 1 2 samples.

1972. Norit GSX was obtained from N V Algemeene Norit, Amersfoort, Holland, and reagents for t assay of lactate and pyruvate from Boehri ger Ma nheim, FRG and for the assay of glucose from Kab Stockholm, Sweden. Adrenaline, oradrenaline and dopamine were obtained as hydrochlorides from Sigma St Louis, USA 6-hydroxy-dopamine was kindly supplied by AB Astra, Sodertälje, Sweden, and bepen by AB V trum, Stockholm, Sweden. AG 1 8 and AG 50 8 ion-exchange resins (Bio Rad, Richmond USA) were extensively washed before use Other chemicals were reagent grade from ordinary commercia suppliers.

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Effect of demedullation and sympathectomy

The effect of demedullation and sympathectomy on mean arterial blood pressure and o arterial plasma catecholamine levels is shown in Table I The blood pressure before bleedin was significantly reduced in all groups of treated animals compared to the controls (p 0.01) Sympathectomy appeared to have a larger effect on blood pressure than demedullation.

In control animals arterial A NA and DA levels were approximately equal (Table I) In animals kept anesthetized for 4 h but not subjected to bleeding there was an approxi mately 3-fold increase in the level of each catecholamine. Following demedullation plasma A was undetactable, while 6-OH DA treatment had no significant effect on A Th plasma NA levels were reduced by some 40% by either demedullation or sympathectomy and by 65% by the combined treatment. Plasma DA was reduced to trace concentrations by either treatment

The basal levels of plasma cyclic AMP glucose, lactate, pyruvate, glycerol, and FFA in the 4 groups of animals are given in Table II Although there were several statistically significant differences between the basal levels in the treated and untreated rats the absolute magnitude was generally small and the physiological significance is unclear In the control rats glucose, lactate, pyruvate and FFA were increased by just keeping the animals for 4 h.

Arterial concentrations of cyclic A[illegible] plasma lactate pyruvate glycerol and [illegible] in animals [illegible] and subjected to hemorrhage [illegible] The animals were untreated control, demedullated (dem), chemically sympathectomized (6-O[illegible]DA) or demedullated sympathectomized (dem 6-O[illegible]DA). Mean [illegible] SE. Number of experiments within parentheses. Values are given for [illegible] and [illegible] after start of the experiments.

	Control		Dem		6-OH-DA		Dem 6-OH-DA	
	0	4 h	0	4 h	0	4 h	0	4 h
Cyclic AMP nmol/l	15 1 2 0 (28)	14 0 2 0 (5)	30 5 ± 7 0^{c} (22)	12.7 ± 7 8 (5)	28 1 ± 6 6^{c} (8)	26 8 ± 10 0^{b} (4)	10 8 ± 2 3^{c} (12)	8 2 ± 2.7^{c} (5)
Glucose mmol/l	89 0 26 (35)	8 77 0 52** (7)	6.23 ± 0 41 (25)	5 20 ± 0 37^{c} (4)	5 05 ± 0.[illegible]^{c} (8)	6.25 ± 0 48^{c**} (4)	7.39 ± 0.14^{c} (11)	7.36 ± 0.24^{c} (5)
Lactate mmol/l	0.94 ± 0 04 (44)	1 19 [illegible] (8)	1 52 ± 0 15^{c} (25)	1.80 ± 0 24^{c} (6)	0 92 ± 0 09 (8)	0 92 ± 0 08 (4)	0 73 ± 0 07^{c} (12)	0.56 ± 0 04 (5)
Pyruvate μmol/l	65 ± 4 (35)	137 ± 86 (5)	67 ± 8 (25)	99 ± 16 (6)	58 ± 12 (8)	54 ± 30 (4)	80 ± 5^{c} (11)	75 ± 4 (4)
Glycerol mmol/l	0 20 ± 0 01 (41)	0 22 ± 0 06 (7)	0 17 ± 0 01 (25)	0.18 ± 0 07^{a} (4)	0 13 ± 0 01^{a} (8)	0.15 ± 0 003^{b} (4)	0 70 ± 0 01 (12)	0.30 ± 0 02^{b*} (5)
FFA mmol/l	1 04 ± 0.20 (10)	1 44 ± 0.33 (4)	0.75 ± 0 06^{c} (25)	0 79 ± 0 13^{c} (5)	1 24 ± 0 11^{a} (8)	1.87 ± 0 49** (4)	—	—

Significantly different from corresponding control (p 0 05).
Significantly different from corresponding control (p 0 01).
Significantly different from corresponding control (p 0 001).
Significantly different from 0-time value (p 0 05).
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6-OH DA	0 h	93±3 (18)	0 71±0.21 (10)	0.68±0.246 (10)	TA (10)
Demed + 6-OH DA	0 h	90± (22)	TA[b] (10)	0.42±0.07 (10)	TA[b] (10)

[a] Not detectable
[b] Trace amou ts found in 1 samples.

197? Nont GSX was obtained from N V Algemeene Nont, Amersfoort, Holland, and reagents for the assay of lactate and pyruvate from Boehringer Mannheim, FRG and for the assay of glucose from Kabi Stockholm, Sweden. Adrenaline noradrenaline and dopamine were obtained as hydrochlorides from Sigma St. Louis, USA. 6-hydroxy-dopamine was kindly supplied by AB Astra, Södertälje Sweden, and heparin by AB V trum, Stockholm, Sweden. AG 1 8 and AG 50 8 ion-exchange resins (Bio Rad, Richmond, USA) were e tensively washed before use. Other chemicals were reagent grade from ordinary commercial suppliers.

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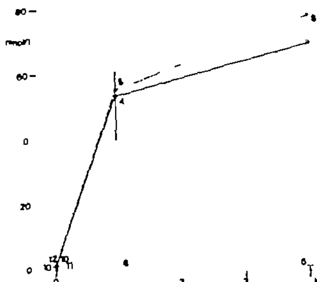


Fig. 2. Effect of bleeding on plasma adrenaline. ●—● Controls. ○—○ Demedullated. — Sympathectomized. □—□ Demedullated and sympathectomized. Mean ± SE. Number of observations are given.

The NA levels rose about 3-fold 1 h after bleeding. This early rise was not altered by either demedullation or by sympathectomy alone (Fig. 3), but prevented by combined demedullation and sympathectomy. In intact rats there was a further 5-fold increase in NA levels between 1 and 4 h after bleeding. Interestingly this secondary rise in NA levels was prevented by demedullation, but not by sympathectomy.

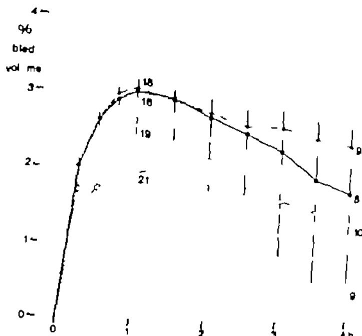
The changes in DA levels following bleeding are illustrated in Fig. 4. There was no change in plasma DA during the first hour of hypotension. On the other hand, between 1 and 4 h after bleeding there was approximately a 20-fold increase, which was prevented by neither demedullation nor sympathectomy alone, but significantly reduced by the combination.

Heart catecholamines

The effects of 6-OH-DA-treatment, adrenal demedullation and bleeding on heart tissue catecholamine levels are presented in Table III. In control animals there was a significant increase in tissue A levels following bleeding, but no significant changes in NA and DA levels. Following demedullation no A could be detected in the heart. In these animals there was a significant ($p < 0.05$) fall in tissue NA content and rise in tissue DA content between 1 and 4 h following bleeding. In the sympathectomized rats the levels of all 3 catecholamines were reduced but a small rise in tissue A levels during the hypotensive period could still be appreciated.

Plasma cyclic AMP

There was a highly significant increase in plasma cyclic AMP levels in all groups of animals following bleeding (Fig. 5 a). The increase in cyclic AMP after 1 h was not significantly altered by 6-OH DA treatment, but was significantly reduced by demedullation and still further reduced by combined demedullation and sympathectomy. After 1 h the levels of cyclic AMP tended to fall. In the intact and sympathectomized animals the levels were



F g 1 Bled volume Rats were connected to a reservoir with a pressure of 35 mmHg. Bled volume is expressed as per cent of body weight. ●—● Control. ○—○ Demedullated. — Sympathectomized □—□ Demedullated and sympathectomized Mean ± $S_{\bar{x}}$ Number of animals at 1 and 4 h are given. In the demedullated group some rats took back both their whole bled volume as well as saline from the reservoir

The same pattern was not seen in the treated rats and may therefore be related to the increase in plasma catecholamines that occurred during the 4-h period in the control group but was not present in the treated animals

Blood volume loss and heart rate When the animals were allowed to bleed into the reservoir against a pressure of 35 mmHg, there was an initial rapid blood loss so that between 1 and 2% of the body weight was lost in 15 min. Thereafter bleeding proceeded at a lower rate for about 45 min (Fig. 1) The maximal amount of blood lost was unchanged by sympathectomy but reduced by about 20% by demedullation and by about 30% by combined demedullation and sympathectomy After about 1 h of hypotension the animals started to take back fluid from the reservoir This decompensation phase was reduced by sympathectomy whether the rats were demedullated or not.

In all groups of animals there was a transient bradycardia. It was maximal 5–10 min after the start of bleeding and thereafter the heart rate returned towards control The reduction of heart rate was unchanged by adrenal demedullation, but significantly reduced by 6-OH DA treatment

Plasma catecholamines

Following 1 h of hypotension the plasma A levels had increased to about 50 nmol/l A further increase to 70 nmol/l occurred after 4 h of hypotension (Fig.). The A response was unaltered by chemical sympathectomy but completely eliminated by adrenal demedullation (Fig 2)

Table III. Catecholamine levels in heart subjected to hemorrhage. The animals were untreated (control), demedullated (dem), chemically sympathectomized (6-OH DA) or demedullated and sympathectomized (dem + 6-OH-DA). An unbled control was also included. Mean ± SE. Number of experiments within brackets. The values are expressed as μmol/kg.

Time	Amine	Control Not bled	Control Bled	Dem	6-OH-DA	Dem + 6-OH DA
1 hr	Noradrenaline		6.61 ± 0.20 (4)	7.08 ± 0.30 (6)	0.55 ± 0.05 (5)	0.49 ± 0.01 (4)
	Adrenaline		0.85 ± 0.17 (4)	ND (6)	0.09 ± 0.01 (5)	ND (4)
	Dopamine		0.05 ± 0.01 (4)	0.07 ± 0.01 (6)	0.06 ± 0.04 (5)	0.02 ± 0.01 (4)
4 hr	Noradrenaline	6.51 ± 0.51 (4)	5.58 ± 0.32 (4)	4.87 ± 0.84 (4)	0.52 ± 0.04 (5)	0.53 ± 0.03 (5)
	Adrenaline	0.20 ± 0.01 (4)	2.96 ± 1.12 (4)	ND (4)	0.23 ± 0.01 (5)	ND (5)
	Dopamine	0.32 ± 0.15 (4)	0.05 ± 0.01 (4)	0.32 ± 0.06 (4)	ND (5)	0.03 ± 0.01 (5)

ND = not detectable.

of cyclic nucleotide phosphodiesterase in plasma of rats (cf Broadus 1977). The content of radioactivity in kidney and liver of the shock animals was lower than that of the intact animals. Thus, in kidney there was radioactivity corresponding to 1.73 ± 0.08 nmol/g in the control group and 1.27 ± 0.08 nmol/g in the shock group (n = 3). In the liver the corresponding figures were 0.39 ± 0.05 and 0.23 ± 0.02 nmol/g. These results taken together suggest that the volume of distribution of cyclic AMP was reduced significantly in shock, but the rate of elimination was not.

Arterial metabolite levels

Plasma glucose (Fig. 5 b) showed principally the same pattern as the cyclic AMP. Thus, in control rats, the levels increased during the early part of the hypotensive period, but were reduced below control values in later stages of hypotension. 6-OH DA treatment tended to raise rather than reduce the plasma glucose levels. On the other hand, demedullation alone or in combination with sympathectomy prevented the rise in glucose. The secondary fall in glucose levels was not prevented, however.

The five- to 6-fold rise on blood lactate levels (Fig. 5 c) in the control rats subjected to bleeding was similarly unaffected by chemical sympathectomy but was blunted by demedullation with or without 6-OH-DA. However, even in the rats subjected to combined demedullation and sympathectomy the rise in lactate was 4-fold, above pre-bleeding values.

The arterial pyruvate levels also rose in bled rats, albeit less than the lactate concentration (Fig. 5 d). In the control rats a 3–4-fold increase was seen. This rise was decreased both

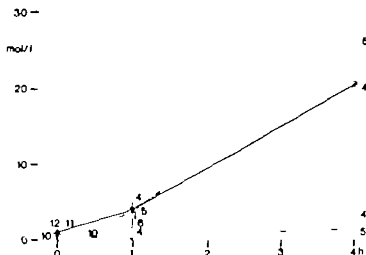


Fig. 3 Effect of bleeding c plasma noradrenaline. ●—● Controls. O—O Demedullated. — Sympathectomized. □—□ Demedullated and sympathectomized. Mean ± SE. Number of observations are given.

still above control after 4 h of hypotension (Fig. 5 a) However in the demedullated anima cyclic AMP was almost undetectable.

In order to determine to what extent the increase in cyclic AMP may be due to decrease plasma volume and to altered elimination, the fate of i v administered H-cyclic AMP wa determined. Following injection of H-cyclic AMP (20 nmol) the plasma level of H-radio activity was approximately 4 times higher in bled animals than in control animals given th same amount (Fig. 6) However the rate of disappearance of cyclic AMP was similar i shocked and control rats, since the ratio between H-cyclic AMP in the two groups o animals remained at about 4 over the entire 10-min period sampled. On the other hand, th disappearance of total radioactivity from plasma was reduced in shock. The rate of disap pearance of nucleotides other than cyclic AMP and of nucleosides and bases was faster i the control animals than in animals subjected to hemorrhage (Fig. 6) Therefore, the reduc rate of H-disappearance following H-cyclic AMP injection in bled rats does not seem t be due to reduced cyclic AMP hydrolysis, but rather to reduced disappearance of the break down products. These findings are therefore in agreement with the presence of high activi

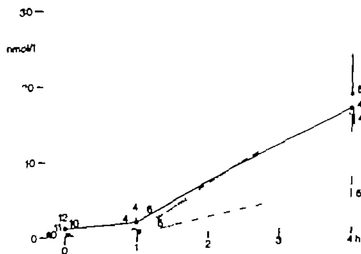


Fig. 4. Effect of bleeding plasma dopamine. ●—● Controls. O—O Demedullated. —× Sympathectomized. □—□ Demedullated and sympathectomized. Mean ± SE. Number of observations are given.

Table III Catecholamine levels in heart subjected to hemorrhage. The animals were untreated (control), demedullated (dem), chemically sympathectomized (6-OH DA) or demedullated and sympathectomized (dem + 6-OH-DA). A nonbled control was also included. Mean ± SE. Number of experiments within brackets. The values are expressed as µmol/kg.

Time	Amine	Control		Dem	6-OH DA	Dem + 6-OH DA
		Not bled	Bled			
1 hr	Noradrenaline		6.61 ± 0.20 (4)	7.08 ± 0.30 (6)	0.55 ± 0.05 (5)	0.49 ± 0.01 (4)
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	Adrenaline	0.20 ± 0.01 (4)	2.96 ± 1.12 (4)	ND (4)	0.23 ± 0.01 (5)	ND (5)
	Dopamine	0.32 ± 0.15 (4)	0.05 ± 0.01 (4)	0.32 ± 0.06 (4)	ND (5)	0.03 ± 0.01 (5)

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The arterial pyruvate levels also rose in bled rats, albeit less than the lactate concentration (Fig. 5d). In the control rats a 3–4-fold increase was seen. This rise was decreased both

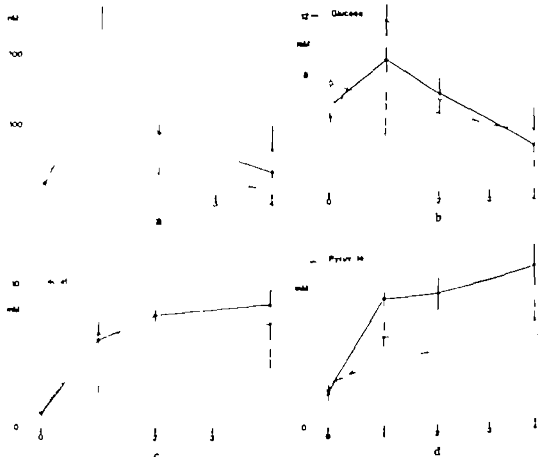


Fig 5 Effect of bleeding on a) plasma cyclic AMP b) plasma glucose, c) blood lactate and d) blo[od] pyruvate. ●—● Controls. ○—○ Demedullated. Sympathectomized. □—□ Demedullated a[nd] sympathectomized. Mean ± $S_{\bar{x}}$ 4–35 observations.

by sympathectomy and by demedullation and was almost prevented by the combined trea[t]ment.

The lactate/pyruvate ratio may be taken as a rough indicator of the oxygen debt (Huck[a]bee 1958) At 4 h after hemorrhage it was 30 in the control rats, 24 in the demedullated rat[s] 40 in the 6-OH DA treated rats and 20 in the rats given the combined treatment. In th[e] unbled control animals the corresponding values were 9 18 17 and 7 respectively Thu[s] in all groups of animals there was an increase in lactate/pyruvate ratio but it was less pro[]nounced in the demedullated rats.

Finally the lipid metabolites, glycerol and FFA showed the pattern of response reporte[d] earlier for the anesthetized rat (Farnebo *et al* 1977) Thus, the glycerol levels were essentiall[y] unchanged, or even in the case of the 6-OH DA treated group decreased 1 h after bleedin[g] (not shown). Four hours after bleeding there was a slight increase over basal in the contro[l] group (to 0.38 ± 0.06 nmol/l), but not in either of the groups of treated rats. The FFA level[s] were reduced to approximately half following 1 h of hypotension and remained low

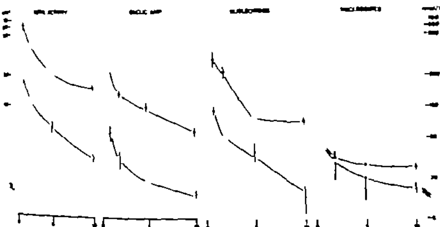


Fig. 4. Fate of ^{3}H-cyclic AMP in control (●—●) bled (○—○) Mean ± SE (= 5). For experimental details see Methods section.

Discussion

We have used determinations of plasma catecholamines to estimate the rates of secretion. The methodological analysis presented elsewhere (Hallman *et al.* 1978), shows that the present method is applicable for determination of catecholamines in rat plasma. A problem is that the plasma catecholamine levels may be affected by alterations in the rate of disappearance or in the size of the miscible central pool. However results obtained in the dog (Watts 1965) and rat (Ferrebo *et al.* 1978) show that the rate of disappearance of plasma catecholamines is similar in bled and non-bled animals. All 3 catecholamines, A, NA and DA, are removed by similar mechanisms. Therefore our finding that A levels may increase 100-fold at a time when DA levels are unchanged is further evidence that the increases in plasma catecholamine levels in shock may be largely due to increased rates of secretion. Alterations in the access to the central blood volume from which samples were taken could be a source of some error. The adrenal medulla probably has ready access to the central blood compartment. At the same time inactivation of A by uptake into sympathetic nerves may be impaired due to decreased peripheral circulation. Furthermore, the majority of NA secreting sympathetic nerves are less close to the central compartment and a greater proportion of their released NA may be taken back into the nerves during bleeding than before. Therefore, the possibility exists that one may overestimate the role of the adrenal medulla if determination of the relative A and NA levels is the sole criterion. However we feel that qualitatively if not quantitatively measurements of plasma catecholamines with the present technique do reflect their rates of secretion.

Bleeding induced a very marked (almost 100-fold) increase in plasma A levels after 1 and 4 h. The increase in NA levels was moderate (3–4 times) during the first hour but large (30-fold) after 4 h of hypotension. The DA levels were unchanged during the first hour of hypotension but reached levels similar to those of NA after 4 h.

Demedullation completely eliminated the rise in A levels following bleeding, which shows that there is practically no contribution of this amine from extra-adrenal sources. The

source of the plasma NA during hypotension is more difficult to define. After demedu tion NA levels rose by 3–4 times 1 and 4 h after bleeding. This rise was almost compl prevented by chemical sympathectomy Thus, sympathetic nerves appear to make a contr tion of this magnitude to the increase in plasma NA On the other hand, animals with che cal sympathectomy showed as large an increase in plasma NA as the intact controls after bleeding. Since a shift from A to NA secretion following prolonged stimulation of adrenal medulla has been described (Kajihara *et al* 1977) this could mean that the large late increase in plasma NA is exclusively of adrenal origin. However the contribu of the adrenal medulla to the plasma NA level may be over-estimated in the animals w lack the neuronal uptake mechanism for NA inactivation. Therefore it is probably saf conclude that the plasma NA levels in late shock derive from both nerves and adr medulla

During the later phase of shock there was also a very large increase in plasma DA le The origin of this DA is unclear It was not reduced by either sympathectomy or by adre ectomy alone Combined sympathectomy and demedullation reduced the increase in l but did not eliminate it. It is therefore likely that adrenal medulla, sympathetic nerves possibly other sources all contribute. Possibly prolonged activation of sympathetic ne and adrenal medulla activates the tyrosine hydroxylase to such a high level of activity t the dopamine β-hydroxylase reaction becomes rate limiting.

Bleeding produced extensive changes in the level plasma cyclic AMP and of circulati metabolic substrates in the anesthetized rat as described earlier (Farnebo *et al* 1977). order to get some idea of the relative importance of circulating amines from the adre medulla and noradrenaline released locally from sympathetic nerve endings we have co pared the responses to bleeding in untreated, demedullated and chemically sympat tomized rats. It must be emphasized that the results were obtained in *anesthetized ani* and the conclusions reached here apply to this condition. Indeed, we have found that th are marked differences in the responses in anesthetized and non-anesthetized rats (Farn *et al* 1978).

The rise in cyclic AMP levels following bleeding was markedly reduced by adrenal medullation, suggesting that endogenous A is more effective than endogenous NA in rai cyclic AMP as has been shown for exogenous amines (Broadus 1977) Even in the demed lated rats and in the rats with combined sympathectomy and demedullation cyclic A was elevated 3–4 times 1 h after bleeding. This could mean that some stimulating fac other than catecholamines, such as glucagon (Broadus *et al* 1970, Jerums *et al.* 1977), responsible for this rise. Although glucagon levels are elevated in shock (Halmagyi *et* 1969) there is increasing evidence that physiological concentrations of glucagon are sufficient to increase plasma cyclic AMP levels (*cf* Broadus 1977). On the other hand, data with H-cyclic AMP suggest that the 4-fold rise in plasma cyclic AMP in the medullated rats may simply be due to a decreased distribution volume

Conversely the data strongly suggest that adrenal A is responsible for the initial rise plasma cyclic AMP in intact and sympathectomized rats subjected to bleeding. It is theref interesting to note that in spite of continued presence of A (and NA and DA) after 1 h hypotension there was a progressive fall in the cyclic AMP content in plasma. An unspec

a dysfunction of the enzyme or a gradual decline in the content of ATP the substrate for cyclic AMP production, are possible explanations. A fall in tissue ATP contents during shock has been reported in several tissues of many species (Blackwood *et al.* 1973, Chaudry *et al.* 1974, McArdle *et al.* 1975, Fredholm and Fronek 1974) Moreover Chaudry and coworkers (1977) recently reported that systemic treatment with ATP $MgCl_2$, which restores tissue ATP levels, also enhanced tissue cyclic AMP levels.

There are certain similarities between the response of plasma glucose and of cyclic AMP to hemorrhage. Thus, in the intact animals there is a rapid increase, followed by a progressive fall, down below control values. The initial increase was enhanced by sympathectomy and reduced or eliminated by demedullation. These findings suggest that in contrast to the situation in cats (Järhult 1975) sympathetic nerves do not play an important role in the mobilization of glucose in rats subjected to hemorrhage.

Blood levels of lactate and pyruvate also rise during hemorrhage. The time course of the rise is different from that observed for glucose, which suggests that it is not only caused by increased glucose levels. The increase in lactate was considerably attenuated by demedullation, but was essentially unaffected by 6-OH DA treatment. The rise in pyruvate was diminished by demedullation as well as by chemical sympathectomy and particularly by the combination. This suggests that at least part of the rise in lactate and pyruvate is due to actions of catecholamines. However also in the rats with combined demedullation and chemical sympathectomy there was a rise in lactate and pyruvate, and in the lactate/pyruvate ratio. Although the influence of endogenous catecholamines is not completely eliminated by the combined treatment the data suggest that the hypotensive shock *per se* alters the lactate and pyruvate metabolism. Studies of anaphylactic shock in rabbits also suggested that lactate and pyruvate levels might change without the mediation of catecholamines (Fredholm and Strandberg 1975). It has been suggested that lactate and pyruvate levels in plasma may be indicators of the severity of shock (Weil and Afifi 1970). The present results indicate that the relationship may be less than perfect. For example, adrenal demedullation, which is known to decrease survival (Chien 1967) decreased rather than increased lactate and pyruvate levels.

During the later phases of hypotension the animals took back fluid from the reservoir. This is usually ascribed to decompensation, leading into an irreversible phase of shock. The reasons for this irreversibility has been much speculated over and one possibility is that it is due to a failure of the sympatho-adrenal system (*cf* Chien 1967). The present results clearly indicate that decompensation may occur even if the secretion of catecholamines is kept high. Similarly Farnebo and Hamberger (1977) found little evidence that sympathetic nerves in shock have a deficient capacity to take up or release NA in shock. Thus a failure of the sympatho-adrenal system *per se* is an unlikely explanation for irreversibility at least in anesthetized rats.

It is interesting that the decompensation phase following bleeding is characterized by low cyclic AMP and glucose levels in plasma. Furthermore, during this late phase of hypotension the FFA levels are depressed. Thus the level of metabolic substrates is low and there is evidence that the mechanisms supporting increased metabolism (*e.g.* cyclic AMP) are also depressed. Therefore the failure of the homeostatic mechanisms during shock may

be due to failing metabolism rather than failing sympatho-adrenal activity Ultimately metabolic failure might (via e g ATP-depletion and acidosis) lead to a failure in the responsiveness to catecholamines

The rate at which fluid was taken back during the decompensation phase was reduced by sympathectomy irrespective of whether the adrenal medulla was present or not. This finding could suggest that in fact the sympathetic nervous system contributes to the irreversible alterations leading to irreversible shock. This is interesting in view of the considerable experimental evidence that pretreatment with α-adrenergic blocking agents may be beneficial in preventing irreversible shock (see Chien 1967) There are at least two explanations for the apparently beneficial effect of combined demedullation and sympathectomy and of pretreatment with α-adrenoceptor antagonists. Firstly the blood loss is reduced. Secondly the degree of peripheral vasoconstriction may be reduced with consequent better tissue perfusion. If the differences in bled volume at 1 hr are taken into account, the most prominent feature during the late phase of bleeding is the remarkable decompensation in the demedullated animals. This is probably due to the combined action of long standing vasoconstriction due to the active sympathetic nervous influence and lack of metabolizable substrate leading to severely compromised tissue function.

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of the content of a vesicle. Nevertheless, if the vesicles were in open communication with the synaptic cleft during the transmission process, both total and partial emptying of a vesicle could result in a parallel release of all components stored in it, such as NA, ATP, protein and sulphomucopolysaccharides (SMPSs).

Stjärne *et al* (1970) reported that stimulation of the splenic nerve of the cat resulted in a release of NA but not of ATP. Such a selective release of NA cannot be explained by a simple theory of complete exocytosis. *In vitro* studies in our laboratory have shown that vesicle material from adrenomedullary cells (Uvnäs and Åborg 1977) and splenic nerves (Uvnäs *et al*., to be published) possesses properties of a cation exchange material capable of binding catecholamines even in the absence of ATP. These findings suggest the possibility that a selective fractionate release of the noradrenergic transmitter might be due to a nerve impulse-induced cation exchange over the depolarized nerve terminal membrane (Uvnäs 1973). As one step on our way to clarifying the question of a selective transmitter release we decided to study quantitative changes of the noradrenergic vesicle components NA, ATP and SMPSs in subcellular fractions from the cat spleen during splenic nerve stimulation. We found that nerve stimulation in the presence of phenoxybenzamine and imipramine induced a significant fall of NA contents in the noradrenergic vesicle fractions but did not lower the contents of ATP and ^{35}S-labelled SMPSs.

Methods

Experimental. Cats of both sexes (2.5–4 kg) were injected subcutaneously with $Na_2\,^{35}SO_4$ (carrier-free, [illegible], Mersey) in a dose of 8 mCi/kg. They were anaesthetized 24 h later with sodium pentobarbital (30 mg/kg i.p.) or chloralose (90 mg/kg i.v.). The body temperature was kept at 36–37°C by means of a heating pad. Heparin (400 I.U./kg) was given i.v. Blood pressure was recorded from the common carotid artery via a Statham transducer on a Grass polygraph. The abdomen was opened by a midline incision. The vessels to the narrow (frontal) portion of the spleen were ligated and the spleen was clamped tightly at the borderline between the narrow and wide (dorsal) portion. The frontal portion—serving as control—was then removed, cut into small pieces, rinsed with ice-cold sucrose, blotted dry and put on ice. Phenoxybenzamine (PBA), (Dibenzyline, Smith, Kline and French), 10 mg/kg, and imipramine (Tofranil, Geigy), 1 mg/kg, were injected i.v. during 20 min each. The splenic nerve was prepared for stimulation. Electrical stimulation of the splenic nerve was carried out with shielded platinum electrodes connected to a S4D Grass stimulator, with supramaximal pulses of 2 ms duration at a frequency of 10 Hz. During 30 min a total of 18 000 pulses was applied. Immediately thereafter the remaining portion of the spleen was removed and treated in the same way as the control. The clamped piece was discarded.

Subcellular fractionation. Both portions of the spleen were subjected to the same fractionation procedure. They were chopped and homogenized in 5 volumes of ice-cold 0.25 M sucrose, containing 0.001 M $MgCl_2$ and 0.005 M potassium phosphate buffer pH 7.4, in an Ultra Turrax apparatus at 100 V for 1 min. The homogenates were centrifuged at 15 000 g_{max} for 15 min and the resulting supernatants were layered onto discontinuous gradients of 16%, 12%, 8%, 4% and 2% Ficoll (Pharmacia, Uppsala) in 0.25 M sucrose. The bottom pellet and 5 bands, visible at the borderlines between the different Ficoll solutions, were collected together with the adjacent Ficoll, diluted with isotonic sucrose and centrifuged at 125 000 g_{max} for 30 min. The final pellets were resuspended by hand in 0.25 M sucrose. The particulate fractions are termed as gradient fraction 1–6 (starting at the top of the gradient). Aliquots of the fractions were taken for determination of NA, ATP, protein, total and lipid-bound ^{35}S, in a few experiments also for cytochrome-C-oxidase (Cy-C-Ox) and glucose-6-phosphatase (G-6-pase).

Assays. NA was extracted from tissue fractions with 0.4 N $HClO_4$, separated by chromatography on Al_2O_3 and assayed by the trihydroxyindole method (Udenfriend 1962). The recovery of NA was 95–103%. Protein was determined according to Lowry *et al.* (1951). ATP was extracted from the fractions by boiling in 0.25 M sucrose for 10 min in a water bath. The precipitated protein was spun down and the ATP deter

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Effect of splenic nerve stimulation on the contents of noradrenaline, ATP and sulphomucopolysaccharides in noradrenergic vesicle fractions from the cat spleen

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Abstract

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In order to assess the effect of splenic nerve stimulation on the contents of noradrenaline, ATP and ^{35}S labelled compounds in noradrenergic vesicles from the spleen, experiments were carried out on the cat spleen *in situ* its frontal portion being used as control Particulate fractions containing noradrenergic vesicles were isolated on Ficoll gradient centrifugation of homogenates from both portions of the spleen The cats received phenoxybenzamine (10 mg/kg) and impramine (5 mg/kg) to inhibit the contractile response of the spleen to nerve stimulation and the reuptake of noradrenaline (NA) into nerve terminals Administration of the drugs did not significantly affect the levels of NA, ATP and ^{35}S (presumably in sulphomucopolysaccarides, SMPSs). In response to prolonged stimulation of the splenic nerve a significant loss of NA from noradrenergic vesicles occurred, whereas levels of ATP and ^{35}S-labelled compounds remained unchanged. The results indicate that a discharge of NA in response to nerve stimulation is not accompanied by simultaneous discharge of ATP and SMPSs

According to current opinion, transmitter release at nerve terminals occurs as a nerve impulse-induced quantal secretion. It has been proposed (for references see Hubbard 1970) that the released quantal package corresponds to the content of a transmitter storage vesicle However comparative calculations of the transmitter content in varicosity vesicles and the amount of transmitter released per nerve impulse have cast doubts on the validity of a simple vesicle theory especially as regards noradrenergic transmission. For example nerve impulse-induced output of noradrenaline (NA) from a varicosity in the rat iris has been estimated to correspond to only a small fraction (400 molecules of NA) of the content of one vesicle (15 000 molecules of NA) out of 1 000 vesicles in the varicosity (Folkow *et al.* 1967). If these calculations are correct, the transmitter quantum might be only a fraction

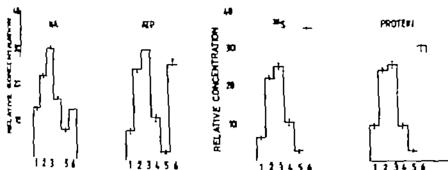


Fig. 1 Distribution of NA, ATP ^{35}S and protein on Ficoll gradients. Particulate fractions of splenic homogenates were isolated on Ficoll gradient centrifugation from control portions of the spleen (=14), described in Methods. NA, ATP ^{35}S and protein in fractions 1–6 are expressed as percentages of their sum on the gradient. Means ± S.E. are given.

Results

Distribution of NA, ATP ^{35}S, protein and marker enzymes on Ficoll gradients from control portions of the spleen

The particulate gradient fractions 1–6 were examined. Levels of endogenous NA, which presumably reflect the distribution of noradrenergic vesicles, were highest in fractions 2 and 3 (of the total), while fractions 1 and 4 contained only about 15% each (Fig. 1 a). The distribution of ATP total ^{35}S and protein were similar to the NA pattern (Fig. 1 b–d). The bottom pellet (fraction 6) with high contents of ATP ^{35}S and protein, was macroscopically inhomogeneous due to heavy conglomerates of particles. This fraction also showed the highest contamination with Cy-C-Ox (Fig. 2 a), while G-6-pase was distributed rather evenly among all gradient fractions (Fig. 2 b). Electron microscopic examination of the fractions (unpublished results) and the distribution of the enzymic markers supported the presumption that the major part of noradrenergic vesicles occurred in fractions 1–4 and was contaminated heavily with microsomes but only slightly with mitochondria.

Lipid-bound ^{35}S represented 20% (fractions 1 2 and 4) and 10% (fraction 3) of the total ^{35}S radioactivity in the corresponding fraction (Table II). In other words, 80–90% of the ^{35}S was incorporated into non-lipid compounds. Therefore, the distribution of total ^{35}S

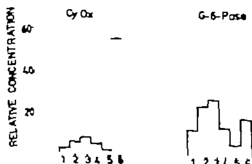


Fig. 2. Distribution of Cy-C-Ox and G-6-Pase on Ficoll gradients. Experimental protocol and presentation of results as in Fig. 1. Total enzymic activities on the gradient were, for Cy-C-Ox 1.35 Δ log ferrocytochrome c/min, for G-6-pase 3.9 nmol inorganic phosphate/min.

mined in the supernatant fluid by the luciferine-luciferase method essentially according to Stanley a Williams (1969) with modifications previously described (Fredholm *et al.* 1977). The extraction (checked t reextractions) was nearly complete. The recovery of ATP in the assay was 94.7±2.6 . The total radioactivity was determined in aliquots of the resuspended fractions after addition of 10 ml Instag (Packard) by liquid scintillation spectrometry in a Packard Tricarb counter. Correction for quenchi was made using an external standard system. Lipid-bound ³⁵S was extracted from the fractions dri overnight over phosphorus pento ide by repeated homogenization and centrifugation in chlorofor methanol (2 1 /v). After evaporation of the solvent, 10 ml of a toluene scintillation liquid were added a the radioactivity due to ³⁵S was determined. Non-lipid bound ³⁵S was expressed as the difference betwe total and lipid-bound ³⁵S.

Cy-C-Ox activity was determined accord ng to Cooperstein and Lazaro (1951). G-6-pase activity assayed by the slightly modified method of Svansson (1955), the inorganic phosphate according to Fur gott and Gubareff (1965).

Statistics Results are expressed as means ± S.E. Hypotheses were tested by Student's t-test.

Comment

The frontal portion of the spleen from the same animal was used as control in order t avoid ariations NA contents of spleens from different cats (Dearnaley and Geffen 1966). While some authors fou differences in sympathetic innervation (Green and Fleming 1968) or NA contents (Dearnaley and Geff 1966) between the th nner and thicker portions, others did not (Hedqvist and Stjärne 1969). In accordan with the latter authors we found equal amounts of total particle-bound NA in the two portions of t organ (F g. 4). Therefore it seems highly preferable to evaluate effects of pharmacological manipulatio and electrical nerve stim lation by comparing portions of the same spleen, nstead of comparing grou of control and treated animals, as done by Chubb *et al* (1972) and by F llenz and Howe (1971).

The homogenates were centrifuged t 15 000 g_{max} for 15 min, in order to d minish contamination noradrenergic vesicles by m tochondria. It has been argued (De Potter *et al.* 197 with Bisby and Fille 1971) that such a centrifugation causes a significant loss of one population of noradrenergic esicles, the dense ones. On checking the yield of noradrenergic vesicles from the rat heart and spleen aft centrifugation in the range 6 000–15 000 g_{max} (unpublished results) we found no significant differences the yields. Neither of course did this centrifugation remove all mitochondria.

W achieved an 18-f ld purification of noradrenergic esicles from the homogenate, with values about 150 ngNA/mg protein. This is not very high compared to maximal values of 720 ngNA/mg prote in fractions isolated from the cat spleen by double gradient technique (Bisby and Fillenz 1971). Sin in our experiments the effects were evaluated by comparing correspond ng noradrenergic vesicle fractio before and after treatment, we considered it more mportant to void a loss of vesicles than to achieve hi purity. Such a loss inevitably occurs during repeated purification procedures (unpublished results).

Drugs were administered before nerve stim latio n order to prevent contraction of the spleen and ree take of released NA. PBA is considered to block postsynaptic α-receptors (Nickerson 1957) as well as the u take of NA into nerve terminals (fo references see Iversen 1967) and, via blockade of presynaptic α-rece tors, to enhance the release of NA and dopamine-β-hydroxylase (DBH) during stimulation (for referen see Stjärne 1976). Since a complete blockade of NA reuptake was desirable and a rise of the PBA dose h a strong depressant effect on the systemic blood pressure, imipramine was used to reinforce the blocka of the reuptake of NA (for references see Iversen 1967). Since the contents of the noradrenergic vesi fractions might be influenced by the drugs, their effect was nvestigated separately.

The influence of drug treatment and nerve stimulation was evaluated by comparing the contents NA, ATP and ³⁵S-labelled compounds i the particulate gradient fractions from the control portion the corresponding values in the fractions from the treated portion of the spleen. Since the amounts tissue in the tw portions were different, absolute values could not be compared. Instead, the amount protein in each fraction was used as reference substance. The amount of the substance (NA, ATP labelled material) per mg protein in each fraction from the dorsal portion was expressed as a percenta of the amount of the substance per mg protein in the corresponding fraction from the frontal portion. Su an evaluation seemed justified, because a) no significant redistribution of protein was observed after dr treatment and nerve stimulation, b) in all experiments the recovery of particle bound protein and N on the gradients from the respective homogenates was approx mately the same for both portions (Table Although a loss of particulate protein from noradrenergic vesicles during nerve stimulation cannot ruled out, such a change appears negligible compared to the total mass of particulate protein in the fra tions.

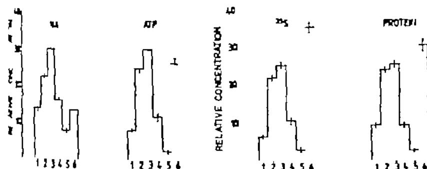


Fig. 1 Distribution of NA, ATP, ^{35}S and protein on Ficoll gradients. Particulate fractions of splenic homogenates were isolated on Ficoll gradient centrifugation from control portions of the spleen (= 14), described in Methods. NA, ATP, ^{35}S and protein in fractions 1–6 are expressed as percentages of their sum in the gradient. Means ± S.E. are given.

Results

Distribution of NA, ATP, ^{35}S, protein and marker enzymes on Ficoll gradients from control portions of the spleen

The particulate gradient fractions 1–6 were examined. Levels of endogenous NA, which presumably reflect the distribution of noradrenergic vesicles, were highest in fractions 2 and 3 (50% of the total), while fractions 1 and 4 contained only about 15% each (Fig. 1 a). The distribution of ATP, total ^{35}S and protein were similar to the NA pattern (Fig. 1 b–d). The bottom pellet (fraction 6) with high contents of ATP, ^{35}S and protein, was macroscopically inhomogeneous due to heavy conglomerates of particles. This fraction also showed the highest contamination with Cy-C-Ox (Fig. 2 a), while G-6-pase was distributed rather evenly among all gradient fractions (Fig. 2 b). Electron microscopic examination of the fractions (unpublished results) and the distribution of the enzymic markers supported the presumption that the major part of noradrenergic vesicles occurred in fractions 1–4 and was contaminated heavily with microsomes but only slightly with mitochondria.

Lipid-bound ^{35}S represented 20% (fractions 1, 2 and 4) and 10% (fraction 3) of the total ^{35}S radioactivity in the corresponding fraction (Table II). In other words, 80–90% of the ^{35}S was incorporated into non-lipid compounds. Therefore, the distribution of total ^{35}S

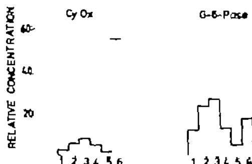


Fig. 2. Distribution of Cy-C-Ox and G-6-Pase on Ficoll gradients. Experimental procedure and presentation of results as in Fig. 1. Total enzyme activities on the gradient were: for Cy-C-Ox, 135 Δ log ferrocytochrome c/min, for G-6-pase 39 nmol inorganic phosphate/min.

TABLE I Recovery of particulate protein and NA on the Ficoll gradient. Particulate fractions of the splenic homogenate were isolated as described in Methods. The sum of protein and NA in all 6 gradient fractions is expressed as a percentage of the total protein in the respective homogenates. F = frontal portion, D = dorsal portion of the spleen. Means ± S.E. are given (n = 23). N.S. = not significant.

	Protein		Noradrenaline	
	F	D	F	D
% of homogenate	2.6 ± 0.2	2.4 ± 0.2	14.7 ± 1.2	N.S. 18.9 ± 1.3

in these fractions may be considered representative for the distribution of non-lipid compounds, presumably SMPSs (and sulphated glycoproteins). It appeared to follow closely the distribution of NA (Fig. 1 c and a).

Influence of drug treatment and splenic nerve stimulation

Three types of experiments were performed: 1) in control experiments both portions of the spleen were removed within an interval of about 1½ h, without any treatment of the cat; 2) in drug treatment experiments PBA and imipramine were administered during this interval; 3) in nerve-stimulation experiments the splenic nerve was stimulated 20–30 min after the end of drug administration.

The percentual changes of NA, ATP and ^{35}S-labelled compounds in gradient fractions 1–4 and in the sum of fractions 1–6 were compared between control and drug-treatment, and drug treatment and nerve-stimulation experiments, respectively (Fig. 3 a–d and 4). Comparison of fractions 5 and 6 was omitted because of low contents of NA and heavy contamination with various subcellular particles, respectively. NA, ATP and ^{35}S contents of corresponding gradient fractions (1–4) from untreated frontal and dorsal portions of the spleen (control experiments) varied between 80 and 120 %. The variations were probably mainly due to methodological inaccuracies during gradient fractionation. After treatment of the animals with PBA and imipramine, no significant changes were observed in the NA and ^{35}S contents of the fractions. Except for fraction 1, there was consistently a slight decrease in the ATP contents of the fractions. In the drug treated spleen, stimulation of the splenic nerve induced a significant reduction of NA to 46–50 % in fractions 1–4 as well as when expressed for the sum of fractions 1–6, but no change in the contents of ATP and ^{35}S-labelled compounds.

TABLE II Contents of lipid-bound ^{35}S in Ficoll gradient fractions 1–4. Cats were injected s.c. with $Na_2{}^{35}SO_4$ 24 h before removal of the spleen. Particulate fractions from the splenic homogenate were isolated as described in Methods. From each fraction lipid-bound ^{35}S was extracted with chloroform-methanol. Lipid bound ^{35}S is expressed as a percentage of the total ^{35}S in each fraction. Means ± S.E. are given (n = 10).

	F 1	F 2	F 3	F 4
% of total ^{35}S	17.5 ± 1.9	13.4 ± 1.8	9.6 ± 0.8	16.4 ± 1.3

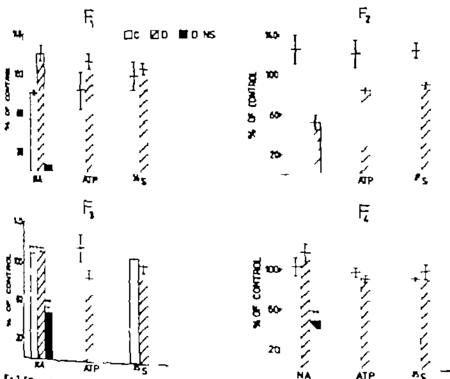


Fig. 3 Effect of drug treatment and nerve stimulation on contents of NA, ATP and ^{35}S in Ficoll gradient fractions F_1–F_4. Particulate fractions containing noradrenergic vesicles were isolated from frontal and dorsal portions of the spleen in control (C), drug-treatment (D) and nerve-stimulation (D + NS) experiments, as described in Methods. PBA 10 mg/kg and imipramine 5 mg/kg were administered i. In both D and D + NS experiments, in the latter the splenic nerve was stimulated with 10 Hz for 30 min. NA, ATP and ^{35}S per mg protein in each fraction from the dorsal portion were expressed as percentages of the corresponding values from the control frontal portion. Means ± S.E. are given. [illegible] of C = 3, [illegible] of D = 4, of D + NS 7. Student's t-test was applied for comparison between C, D and D + NS experiments. C–D, D–(D + NS), C–D–(D + NS). p [illegible] 0.001 [illegible] = 0.0025 $p < 0.01$

The NA/ATP molar ratios (Table III) in gradient fractions from control and drug-treated spleens had values between 1.7 and 4.8. Stimulation of the splenic nerve in the drug-treated [illegible] was followed by a significant fall of the NA/ATP ratios, most pronounced in fraction 4.

The close correlation of the percentual changes in total and non-lipid ^{35}S in the drug-treatment experiments (Table IV), together with the prevalence of non-lipid ^{35}S over lipid ^{35}S in the fractions (Table II), support the assumption that changes of total ^{35}S in the nerve stimulation experiments reflect mainly changes of non-lipid ^{35}S (which for technical reasons were not measured directly). Since no changes of total ^{35}S were observed after nerve stimulation (Fig. 3 a–d, 4) we may assume that the contents of non-lipid ^{35}S were not affected, [illegible]

Discussion

The distribution of noradrenergic vesicles on the Ficoll density gradient (used in order to avoid hyperosmotic media) differed from the pattern usually reported for sucrose gradients. On the latter two peaks of NA were found at 1.0 and 1.4 M sucrose on isopycnic centrifuga-

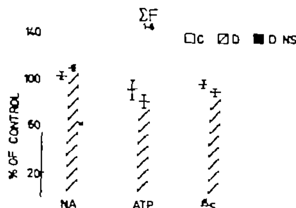


Fig. 4 Effect of drug treatment and nerve stimulation on contents of NA, ATP and ^{35}S in the sum of particulate gradient fractions 1-4. For explanation see Fig. 3

tion of microsomal pellets from the dog spleen (Chubb *et al* 1970), and at 0 7 M and 1 1 M sucrose in the cat spleen (Bisby and Fillenz 1971) In a few experiments we relayered subcellular fractions of the rat spleen from Ficoll gradients onto continuous sucrose gradients fraction 1 sedimented mainly at 0.5 M fractions 2 and 3 at 1 1–1.2 M and fraction 4 at 1.5 M sucrose (unpublished results) Although discontinuous gradients do not provide information as to the equilibrium density it seems that by means of Ficoll gradients several populations of NA-containing particles may be separated, possibly on the basis of other characteristics beside density The existence of several populations of NA-containing particles has been indicated by electron microscopic observations (Tranzer 1973), and by the presence of 3–4 peaks of particulate NA on sucrose gradients, when homogenates from the dog spleen were, after low precentrifugation, directly layered on the gradients (Nelson and Molinoff 1976) These vesicle populations were characterized by different NA/DBH ratios

The concomitant distribution of NA and ATP in fractions 1–4 agrees well with the known presence of ATP in noradrenergic vesicles (for references see Lagercrantz 1976). The parallel distribution of NA with ^{35}S-labelled material in fractions 1–4 confirms our previous findings on various other types of gradients (Åborg *et al* 1972, Blaschke and Uvnäs in Uvnäs 1972) Since more than 80 % of the ^{35}S-labelled material was incorporated into non lipid compounds, *i.e* SMPSs and possibly sulphated glycoproteins (Robinson

TABLE III Effect of nerve stimulation (NS) on the NA/ATP ratio in the Ficoll gradient fractions 1-4 After removal of the frontal portion of the spleen, cats received PBA 10 mg/kg and imipramine 5 mg/kg and the splenic nerve was stimulated with 10 Hz for 30 min. Then the dorsal portion of the spleen was removed Particulate fractions of the homogenates were isolated as described in Methods. NA/ATP ratios from control and drug-treated portions of the spleen are given in row 1 NA/ATP ratios from the control portions (row 2) were compared with the corresponding ratios from the drug treated and nerve-stimulated portion (row 3) by the paired t test. Means ± S.E. are given (= 7). = $p < 0.05$ = $p < 0.01$ = p 0.005.

	F 1	F 2	F 3	F 4
Control and drug treated spleen	4 1±0 4	.0±0 1	2.7±0.2	3.2±0.2
Before NS (= frontal portion)	4 8±1 1	1 7±0.2	1.8±0.1	3.0±0 2
After NS (= dorsal portion)	2.3±0.6	*0.8±0 2	*0 8±0 0	1 5±0 2

TABLE IV. Comparison of the effect of drug treatment on the total and non-lipid ^{35}S in the Ficoll gradient fractions. Cats were injected i.c. with $Na_2{}^{35}SO_4$ and i.v. with PBA (10 mg/kg) and Imipramine (3 mg/kg) 24 h and 2 h before removal of the spleen, respectively. Particulate fractions of the splenic homogenates were isolated, and total and lipid-bound ^{35}S determined as described in Methods. Non-lipid ^{35}S = total ^{35}S – lipid-bound ^{35}S. ^{35}S per mg protein in each fraction from the distal portion is expressed as percentage of the corresponding value from the control (central) portion. Means ± S.E. are given. The effect on total and non-lipid ^{35}S was correlated $r = 0.79$ significant at $p < 0.01$.

	F 1	F 2	F 3	F 4	F 5	F 6
Total ^{35}S	104.1	88.8	96.8	98.1	76.4	80.5
(% of control)	±5.4	±3.1	±6.7	±7.5	±7.3	±5.1
Non-lipid ^{35}S	104.9	78.9	94.3	83.6	78.6	81.6
(% of control)	±19.3	±1.2	±6.6	±7.7	±7.2	±8.7

and Green 1962, Margolis and Margolis 1970), the results further support our previous conclusions that SMPSs are true components of the noradrenergic vesicle (Blaschke *et al.* 1976). The distribution of ^{35}S-labelled lipids corresponded approximately to the pattern of the microsomal marker G-6-pase, in agreement with Lagercrantz (1971), who found more lipids in microsomal fractions than in noradrenergic vesicle fractions. A part of the lipid-^{35}S might be, however, incorporated into sulphatides, which have been shown to be present in noradrenergic vesicles from bovine splenic nerves (Blaschke *et al.* 1976).

Up to 2 h after the i.v. administration of PBA and imipramine, we observed no significant changes of NA levels in the noradrenergic vesicle fractions. The finding is at variance with the results of Fillenz and Howe (1975), who observed a 50% decrease of splenic NA levels in rats treated with PBA. However, their dose of PBA was 2.5 times higher and the time which elapsed after administration of the drug was longer; moreover, since the rats were intact, their sympathetic activity could have been increased reflexively following the α-receptor blockade (Dontas and Nickerson 1957). Our findings are, on the other hand, in good agreement with the observations of Hedqvist and Stjärne (1969), who did not see any rise of the basal outflow of NA from the isolated spleen in the presence of PBA.

The unchanged levels of ATP and ^{35}S-labelled compounds in the noradrenergic vesicle fractions after administration of the drugs indicate the the drugs do not affect these vesicle components.

The 30–55% reduction of particle-bound NA after stimulation of the splenic nerve with 10 Hz for 30 min is fully comparable with a 39% reduction of NA in PBA treated perfused spleens after stimulation with 1–10 Hz for 10–20 min (Hedqvist and Stjärne 1969). On the other hand, in the absence of drugs Euler and Hellner Bjorkman (1955) found no consistent changes in NA contents of the stimulated portion of the spleen in comparison to the unstimulated. It seems that synthesis and reuptake of NA allow the stores of NA to be maintained at approximately constant levels. Chubb *et al.* (1972), stimulating the nerve with 10 Hz for 30 min, did find a reduction of NA of about 27% even in the absence of drugs, but they compared spleens of different cats. As pointed out above, the comparison of spleens of different cats might well give misleading results, especially when the NA/protein ratio is used for estimating the amount of NA in the whole spleen.

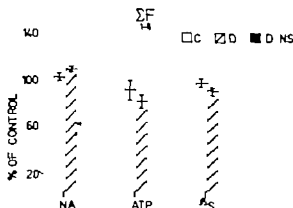


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While in our experiments nerve stimulation induced a 50 % decrease of NA in noradrenergic vesicle fractions, it did not affect their contents of ATP indicating that substantial loss of ATP from the noradrenergic vesicles could have occurred. Also significant decrease of the NA/ATP molar ratios in fractions 1–4 induced by nerve stimu tion supports the contention that NA and ATP are not released together Several obser tions by others favour this conclusion, too Stjärne *et al.* (1970) accordingly found no incre in the outflow of ³²P labelled material from the spleen during stimulation and, sim taneously no decrease of ATP in the splenic capsule, while 50 % of the NA content of spleen was released Potter and Axelrod (1963) observed that a reserpine-induced reduct of NA in rat hearts was not accompanied by a fall of ATP A decrease in ATP conte of adipose tissue induced by nerve stimulation could be prevented by an α-receptor blo ade which eliminated the vasoconstrictor effect of nerve stimulation and thereby the lo hypoxia (Fredholm *et al* 1977) The drop and subsequent increase of ATP in seminal du after sympathetic denervation was considered to be mainly due to changes in effec cells, *i.e* in the smooth muscle energy store (Westfall *et al* 1975)

The NA/ATP ratios of our controls (1 7–4.8) were in the range of similarly puri noradrenergic vesicle fractions from peripheral tissues (Potter and Axelrod 1963 2.9–4 Livett 1968 1.3–3 7) However in vesicle preparations from the bovine splenic nerve tru that were virtually free from ATP-containing contamination, the ratios were 6.8 ± (Yen *et al* 1976) Moreover by extrapolation to *in vivo* conditions Lagercrantz *et* (1975) estimated the ratio to be 19 in the distal end of the splenic nerve and up to in the nerve terminals. They therefore concluded that a major part of NA in the termin is not bound to ATP Consequently one cannot expect it to be released together w ATP in a stoichiometric ratio (4 1) On the other hand, it has been well established th ATP is a powerful mediator of NA uptake (for references see Euler 1970) and may expected to function as such also during the process of reuptake. Recent studies isolated nerve vesicles support this view (Yen *et al* 1973) since the rate of NA relea from the vesicles is retarded in the presence of ATP the reason for the slower net loss of N might be an ATP-facilitated uptake In addition, ATP itself was utilized at a much slow rate than NA was released (Yen *et al* 1976) In view of these findings one might specula that the ATP in noradrenergic vesicles, instead of being an inert binding substance whi passively flows out of the terminal together with NA, has a more dynamic function. Su a function could be the utilization of ATP during the energy requiring process of reu take that helps to maintain the transmitter economy at the nerve terminal. The blocka of the NA reuptake both in our own and in the experiments of Stjärne *et al* (197 would, of course, prevent such a utilization of ATP Hence, the ATP levels will not chang

The non-lipid compounds labelled *in vivo* with ³⁵S-sulphate are presumably SMPSs an possibly sulphated glycoproteins (Robinson and Green 1962, Margolis and Margol 1970) SMPSs have been identified in highly purified noradrenergic vesicles from bovi splenic nerves as well as in noradrenergic vesicle fractions from various sympathetical innervated tissues (Blaschke *et al* 1976) Since drug treatment and nerve stimulation di not diminish the contents of ³⁵S-labelled compounds in any fraction, a significant loss o SMPSs during the release of NA might be excluded

In conclusion: While stimulation of the splenic nerve was followed by a marked decrease of NA contents in the noradrenergic vesicle fractions, the contents of ATP and ^{35}S-SMPSs were not lowered. This finding is highly suggestive as an argument against the conjecture of complete exocytosis, i.e. the release of the entire soluble contents of the NA vesicle. Exocytosis has been defined as a process consisting of a fusion of the vesicular membrane with the plasmalemma, followed by emptying of the soluble contents of the vesicle directly to the exterior of the cell, without passing the cytoplasma (Katz 1969, Pfenninger 1973). While some authors imply that by exocytosis the entire soluble contents run out (Smith 1971, De Potter 1973), others consider the possibility that only a part of the contents come out by a so-called partial exocytosis (Geffen et al. 1970, Stjärne 1976).

Only a fraction of the soluble vesicle proteins is released together with NA during nerve stimulation (Geffen et al. 1970, Smith et al. 1970, Weinshilboum et al. 1971, Gewirtz and Kopin 1970). Moreover, the major mechanism for the "short-term" maintenance of NA in nerve terminals is an almost complete reuptake of the liberated NA (Hedqvist and Stjärne 1969). Such highly economic behaviour of the neuron is difficult to reconcile with a continuous loss of the total soluble part of the NA vesicle unless we assume that there is a reuptake of the macromolecular compounds. Our results are compatible with the concept of a partial exocytosis with a selective release of the transmitter. ATP may be utilized in the process of NA reuptake.

The mechanism of the ATP-independent storage and release of NA remains unclear. Uvnäs (1973) speculated upon the existence of a functional transmitter pool in the nerve terminal vesicles with properties of a cation exchanger. The nerve impulse-induced transmitter release should be due to a cation exchange between the NA in the functional transmitter pool and inorganic ions in the neuro-effector junctional gap. The ion exchange should occur at the nerve terminal—vesicle contact area during the period of nerve impulse induced depolarization. In analogy to the heparin-protein complex in mast cell granules (for ref. see Uvnäs 1977), a SMPS-protein complex was suggested as a possible matrix for a functional release pool in noradrenergic vesicles (Åborg et al. 1972, Blaschke et al. 1976). The present finding that SMPSs are not released together with NA on splenic nerve stimulation is in agreement with this hypothesis. Further insight into the mechanism of the ATP-independent storage and release of NA at the nerve terminals might emerge from studies presently going on in our laboratory.

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[illegible] increase the level of tissue cadaverine and such an effect might influence its synthesis. The diamine oxidase activity is known to be high in the placenta and plasma of pregnant rats (Roberts and Robson 1953, Kobayashi 1964).

Methods

Adult female rats, weight 240–340 g, of the Sprague-Dawley strain were used. They were fed standard pellet diet and water ad libitum. Their estrus cycles were followed by vaginal cytology. Smears were taken [illegible] morning and stained with methylene blue. Only rats with normal estrus cycles were used. Rats selected to become pregnant were allowed to mate in the pro-estrus or early estrus phase. They were then transferred to [illegible] male for 24 h. The next morning when sperm was found in the vaginal smear was considered as the first day of pregnancy. On the 19th day of pregnancy the rats were killed by cervical dislocation and exsanguinated. Concomitantly control rats were sacrificed. One group of rats was studied under undisturbed conditions and another group after the injection of the diamine oxidase inhibitor aminoguanidine (20 mg aminoguanidine hemisulphate/kg) for 10 consecutive days before the actual experiment.

Tissues of pregnant rats (ovary, uterus, kidney, liver, fetus and placenta) and of non-pregnant rats (ovary, uterus, kidney and liver) were rapidly removed, cooled on ice, minced with scissors and gently homogenized in [illegible] Dounce type homogenizer in 4 volumes of cold 0.1 M sodium phosphate buffer (pH 7.2) containing 5 [illegible] 10^{-4} M dithiothreitol, 10^{-4} M EDTA and 2% (w/v) glucose. The homogenate was centrifuged at 20 000 g for 20 min at 4°C. The supernatant was used as enzyme source. Supernatant corresponding to 100 mg of tissue (uterus, kidney, liver and fetus), 60 mg of tissue (placenta as its whole, maternal and fetal [illegible]) and 16–57 mg (ovary) was incubated in the presence of pyridoxal-5-phosphate (final conc. 10^{-4} M), DL-(1-^{14}C)-lysine monohydrochloride (final conc. 10^{-4} M, sp. act. 10 μCi/mmole) and the same buffer as used for homogenization, the total finally made up to 1.0 ml. The incubation was carried out at 37°C for 60 min with agitation. The reaction was stopped by the tipping of 0.7 ml of 2 M citric acid from [illegible] side arm of the incubation vessel. Expelled $^{14}CO_2$ was trapped on [illegible] 10 [illegible] 25 mm piece of No. 005 [illegible] filter paper prepared with 100 μl hydroxide of Hyamine (1 M solution in methanol). For maximal absorption of the $^{14}CO_2$ the shaking was continued for another 45 min. After completed incubation the filter paper was dropped into [illegible] scintillation vessel containing 8 ml of scintillation solution (Bray 1960) and the radioactivity was measured in [illegible] Packard Tri-Carb liquid scintillation spectrometer.

For stoichiometric determinations of cadaverine formation supernatants of the placenta and ovary were incubated in the presence of aminoguanidine in [illegible] final concentration of 2 [illegible] 10^{-4} M to prevent oxidative deamination of the formed cadaverine by diamine oxidase. The reaction was stopped by the tipping of 0.7 ml [illegible] sulfosalicylic acid from the side arm of the incubation vessel. The amount of $^{14}CO_2$ expelled was compared with the amount of the cadaverine formed in the same incubate. For the preparation of the sample for analysis of its cadaverine content 0.4 mg EDTA was added to each ml of the incubate. The incubates were then centrifuged at 800 g for 15 min whereafter the samples were adjusted to [illegible] pH value of 2.0–2.5 and filtered through [illegible] filter with [illegible] pore size of 0.22 μm (Millipore Corp.). The separation and quantitative estimation of cadaverine in the incubates were carried out on [illegible] 6 [illegible] 78 mm column of Durrum DC 4A using an automatic amino acid analyser (LKB-BIOCAL, 3201). The procedure as described by [illegible] (1978) was used with minor modifications.

For determination of the content of cadaverine in the tissues of pregnant and non-pregnant rats about 100 mg of tissue was homogenized in [illegible] Duran 50 type homogenizer in 3.5 ml sulfosalicylic acid (40 mg sulfosalicylic acid and 0.4 mg EDTA/ml). The extracts were then boiled on [illegible] water-bath for 30 min, whereafter they were prepared and assayed in the same way as described above for determination of cadaverine content in incubates.

Results

Cadaverine formation in various tissues from pregnant and non-pregnant rats

The formation of cadaverine in vitro in various tissues from pregnant rats was examined at [illegible] [illegible] from an earlier investigation (Andersson *et al.* 1978), the urinary excretion of

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Formation of cadaverine in the pregnant rat

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Abstract

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The formation as well as the content of cadaverine were determined in different tissues of pregnant an non-pregnant rats. The placenta and ovary were most potent in the ability to form cadaverine. To ou knowledge this is the first report of an in vitro formation of cadaverine linked to a normal physiologica process, i.e. pregnancy. The highest concentration of cadaverine was found in the placenta and ovary o the pregnant rat. Treatment with aminoguanidine generally elevated the content of cadaverine, indicatin a role of diamine oxidase as a regulator of diamine content.

Key words: Cadaverine formation, content of cadaverine, rat pregnancy

Evidence of in vitro formation of cadaverine in mammalian tissue was originally reporte in the mouse kidney after the injection of the anabolic steroid Durabolin (Henningsson Persson and Rosengren 1976). An in vivo formation of cadaverine is supported by the observation that rats regularly excrete cadaverine in the urine. The amine has also bee detected in human urine (Bremer and Kohne 1971). In the pregnant rat the urinary excretio of cadaverine together with other diamines as well as the polyamine spermidine is greatl increased (Andersson, Henningsson and Rosengren 1978). On the 15th day of pregnanc the cadaverine excretion began to rise and hence increased continuously to day 19–20 o pregnancy when peak values up to 10 times the non-pregnant level were observed. There-after the cadaverine excretion fell towards the non pregnant level which after the birth o the young was resumed.

The purpose of the present study was to investigate in which tissue(s) of the pregnant ra an increased formation of cadaverine takes place. In addition the content of cadaverine was determined in various tissues of the pregnant and non-pregnant rat. As stated above very little is known about the synthesis of cadaverine in mammalian tissues. Regarding the close congener of cadaverine, i.e. putrescine, it has been shown that its synthesis is controlled by the level of the amine itself in different cell culture systems and in intact animals (for ref see Jänne, Pösö and Raina 1978). The diamine oxidase inhibitor aminoguanidine might a

Table III. Content of cadaverine (nmol/g) in different tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values ± S.E. of mean of 4 determinations, except for those indicated with (a) which stands for 3 determinations. A. untreated rats, B. rats treated with aminoguanidine. 0 stands for not measurable amount.

Tissue	Pregnant A	Pregnant B	Non-pregnant B
Ovary	14 ±4.75	28 ±5.04	4.1±.62
Uterus	3.9±2.44	5.3±1.78	2.7±1.98
Kidney	0	23 ±3.10	32 ±2.71
Liver	7.6±3.00	14 ±4.22	7.9±0.43
Fetus	3.3±3.25	11 ±1.61 ( )	
Whole placenta	10 ±1.43	14 ±2.42	
Fetal placenta	13 ±0.88	15 ±1.20 (a)	
Maternal placenta	9.5±2.65	20 ±3.28 (a)	

*$p<0.05$ and **$p<0.01$ versus pregnant rats treated with aminoguanidine.

Parallel assays of $^{14}CO_2$-production and the formation of cadaverine from DL-(1-^{14}C)-lysine were performed to ensure that the evolution of $^{14}CO_2$ provided a valid measure of the biosynthesis of cadaverine. Values of cadaverine formation in the placenta and the ovary are shown in Table II from which it is apparent that there was a fair accordance in results obtained by the two methods showing that artifactual release of $^{14}CO_2$ did not occur.

Content of cadaverine in various tissues from pregnant and non-pregnant rats

The cadaverine concentration in tissues of pregnant rats was determined. Of these rats one group had been injected with aminoguanidine for 10 days before the actual experiment, the other group was untreated. The results are summarized in Table III in which also results from non-pregnant rats given aminoguanidine are presented. In the untreated pregnant rat most cadaverine was found in the ovary and placenta. Aminoguanidine treatment of the animals generally elevated the cadaverine concentration of the tissues. The most distinct increase was seen in the kidney, an organ known to be a source of diamine oxidase. Also in the non-pregnant group the highest concentration of cadaverine was found in the kidney. Notable is the significant difference in the cadaverine content of ovary between the pregnant and non-pregnant animals, a result which might reflect the high synthesis in the ovary of the pregnant rat.

Discussion

Although it has been known for some time that cadaverine is regularly excreted in the urine its origin is uncertain. Most authors regard the amine as a contaminant believed to be formed by bacteria in the lumen of the intestine, absorbed and carried by the blood to tissues and urine. However some recent findings strongly suggest that cadaverine can be synthesized in mammalian tissues both in vivo and in vitro. Cadaverine has been found in the brain and blood of germ-free mice (Stepita-Klauco and Dolezalova 1974). An in vitro formation of cadaverine in mouse kidney has been observed after the injection of the anabolic steroid

TABLE I Formation of cadaverine (nmol/g × h) in different tissues of pregnant and non-pregnant ra[illegible] The tissues of the pregnant rats were excised on the 19th day of pregnancy The figures are mea[illegible] values ± S.E. of mean of 8 observations (tissues from 4 untreated rats and 4 rats treated w[illegible] aminoguanidine)

Tissue	Non-pregnant	Pregnant
Ovary	25 ±7 47	110 ±16.3
Uterus	2_ ±3 40	9 0± 1 49
Kidney	2.1±1.03	3.9± 1.38
Liver	2.7±2.11	1 8± 1.01
Fetus		5.9± 1 94
Whole placenta		250 ±20.9
Fetal placenta		350 ±29 0
Maternal placenta		5_ ±10.0

– $p < 0.01$ and – $p < 0.001$ ersus non-pregnant.

cadaverine is known to be high, *i.e* on the 19th day of pregnancy In addition, dete[illegible] minations were made on tissues of non-pregnant female rats. The tissues were collected fro[illegible] two groups of rats of which one group was treated with aminoguanidine as states abov[illegible] the other was not Since no difference was found between the values of the two groups, t[illegible] figures are brought together and presented in Table I from which it will be seen that of t[illegible] tissues examined the placenta was most potent in the ability to form cadaverine, followe[illegible] by the ovary and the uterus. As to the uterus the activity was higher in that of the no[illegible] pregnant rat than in that of the pregnant one. This may be attributed to the fact that t[illegible] activity of cadaverine formation is expressed in nmol per g wet weight of the tissue. It [illegible] known that the pregnant uterus is edematous and for that reason the total activity of t[illegible] organ may not be reduced during pregnancy Both kidney and liver showed low cadaveri[illegible] formation and no differences between pregnant and non-pregnant rats were observed.

The question arose as to which part of the placenta, the maternal one and/or the fet[illegible] one, contributed to the synthesis of cadaverine. For that reason the organ was macroscop[illegible] cally dissected and the enzyme activity of the maternal and fetal parts was determine[illegible] It was found that the enzyme responsible for the formation of cadaverine was mainl[illegible] located in the fetal placenta, in fact the synthesis of cadaverine in the maternal part wa[illegible] only 15% of that in the fetal one (Table I).

TABLE II Stoichiometric determinations of cadaverine formation in tissues of the pregnant rat. Parall[illegible] assays of $^{14}CO_2$ production and formation of cadaverine (nmol/g × h) were carried out in the sam[illegible] incubata.

	$^{14}CO_2$	Cadaverine
Whole placenta	340	340
	280	330
	220	180
	200	170
Fetal placenta	410	450
	340	440
Ovary	64	50
	43	39

TABLE III Content of cadaverine (nmol/g) in different tissues of pregnant and non-pregnant rats. The organs of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values ± S.E. of mean of 4 determinations, except for those indicated with (a) which stands for 3 determinations. A: untreated rats, B: rats treated with aminoguanidine. 0 stands for not measurable amount.

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Durabolin (Henningsson *et al* 1976) In the present investigation an in vitro formation of cadaverine was linked to a normal physiological process, *i.e* pregnancy

The high formation of cadaverine in the fetal part of the placenta is reflected in an elevated excretion of cadaverine during the last trimester of rat pregnancy (Andersson *et al* 1978) This circumstance is supported by experiments carried out on the 19th day of pregnancy showing that after the removal of the fetuses without dislodging the placentas a high urinary excretion of cadaverine was maintained, while on removing also the placentas the increased cadaverine excretion was abolished (to be published)

The elevated in vitro formation of cadaverine in the ovary during pregnancy is consistent with an increased endogenous cadaverine content of the organ indicating that cadaverine can be formed in vivo by decarboxylation of lysine.

Our present results together with our earlier findings of a regular pattern of urinary cadaverine excretion during pregnancy and the observation that cadaverine was formed in the mouse kidney stimulated to growth by an anabolic steroid strongly support our opinion that cadaverine is formed by mammalian tissue cells, and is not the product of bacterial decarboxylation of lysine

Aminoguanidine treatment of the animals doubled the concentration of cadaverine in the maternal part of the placenta which is very rich in diamine oxidase. In the fetal part with a low content of diamine oxidase the change in cadaverine content was only slight. It appears conceivable that diamine oxidase could function as a regulator of cadaverine content in certain tissues rich in the enzyme. Another role of diamine oxidase in the maternal part of the rat placenta may be to protect the maternal tissues from diamines derived from the fetuses and/or the fetal part of the placentas.

The results presented here support our view of a connection between diamine metabolism and the hormonal status prevailing in pregnancy and are also another example of elevated diamine formation in relation to growth.

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Prevention of protein extravasation in the brain by an anion transport inhibitor in acute experimental hypertension in rats

By

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Abstract

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Acute experimental hypertension induces protein leakage in the brain. The protein is thought to be, at least to a great extent, transported through the endothelial cells by pinocytosis. An anion transport inhibitor 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid, markedly reduced adrenaline or bicuculline-induced leakage of ^{125}I-HSA and Evans blue-albumin in all areas of the rat brain. The preventive effect of dexamethasone was less pronounced and no effect was seen of haloperidol.

Key words: Acute hypertension, blood brain barrier, protein tracers, pinocytosis, anion transport inhibitor, dexamethasone.

The metabolic and structural integrity of the endothelial cells of cerebral blood vessels is a prerequisite for the maintenance of the blood brain barrier (BBB) to macromolecules. Abrupt rise in arterial blood pressure can result in multifocal areas of protein extravasation. In addition to the degree and rapidity of the blood pressure increase extravasation can be modified by factors that influence the cerebral vascular tone. Vasodilatory anesthetics and drugs as well as hypercapnia increase the protein leakage whereas hypocapnia has a prophylactic effect (Johansson 1976 a, b, Forster *et al.* 1977). Furthermore, some substances such as dexamethasone, reduce protein extravasation by a not clarified mechanism (Eisenberg, Barlow and Lorenzo 1970, Johansson 1978).

The anion transport blocking drug 4-acetamido-4'-isothiocyano-stilbene 2,2'-disulfonic acid (SITS) has recently been demonstrated to prevent the cholera toxin induced intestinal hypersecretion as well as cholera toxin induced activation of adenyl cyclase (Lönnroth, Hansson, Lange and Holmgren, in preparation). The secretion of serotonin from storage sites in human platelets by exocytosis is also inhibited by SITS (Pollard *et al.* 1977).

Previous studies have demonstrated that proteins extravasated in the brain after acute hypertension at least to a great extent are transported thorough the endothelial cells by

TABLE I. Mean arterial pressure (MAP) before and after the blood pressure elevation induced by bicuculline or adrenaline. $PaCO_2$ immediate prior to the pressure rise. Mean value ± S.E. PaO_2 was > 13.3 kPa (100 mmHg) in all rats.

Experimental group	n	Initial MAP mmHg	Maximum MAP	$PaCO_2$ kPa
Bicuculline (1.. mg kg⁻¹)				
Controls	6	142±3	187±5	4.66±0.08
SITS (25 mg kg⁻¹)	6	136±2	185±2	4.92±0.17
Dexamethasone (4 mg kg⁻¹)	6	139±3	196±2	4.65±0.09
Haloperidol (1 mg kg⁻)	6	132±4	189±3	4.91±0.13
Adrenaline (20 μg kg⁻)				
Controls	8	126±6	194±2	4.44±0.10
SITS (25 mg kg)	8	134±3	192±2	5.00±0.06

pinocytosis (Hansson, Johansson and Blomstrand 1975 Hansson and Johansson 1979 Nag, Robertson and Dinsdale 1977 Westergaard van Deurs and Brondsted 1977), *i.e.* a process not normally occurring in cerebral blood vessels.

Transendothelial transfer of vesicles is energy dependent and could theoretically be influenced by SITS The aim of the present study was to test if it was possible to prevent the protein extravasation in the brain caused by acute hypertension by pretreatment with SITS

The blood pressure increase was induced either by adrenaline or by bicuculline. Bicuculline is a GABA receptor blocking agent that induces epileptic activity and hemodynamically gives rise to hypertension and a massive cerebral vasocilatation (Meldrum and Nilsson 1976) An extensive and exclusively pressure dependent BBB dysfunction is observed (Johansson and Nilsson 1977)

For comparison, some rats were pretreated with dexamethasone. It has recently been claimed that chlorpromazine decreases albumin extravasation around cerebral stab wounds (Rosengren *et al* 1978) and the authors postulated a possible effect via inhibition of the adenylate cyclase. In an earlier study we found a preventive effect of haloperidol in amphetamine induced BBB dysfunction which, however in that study was thought to be secondary to an effect on the blood pressure (Carlsson and Johansson 1978). As haloperidol is closely related to chlorpromazine we included a trial with haloperidol.

Material and methods

Male Sprague-Dawley rats weighing 250–350 g were used. Anesthesia was induced with diethyl ether the rats tracheotomized, immobilized with suxamethonium chloride and mechanically ventilated with gas mixture of 30% O_2 and 70% N_2O Cannulae were inserted in one femoral vein (for administration of drugs) and in both femoral arteries (for electromanometric recording of mean arterial pressure, MAP and for sampling arterial blood for blood gas determinations). Body temperature was kept close to 37°C by means of intermittent external heating. Repeated blood gas determinations were made to ensure stable baseline with PaO_2 greater than 13 1 kPa (100 mmHg). SITS (Nutritional Biochemical Co, Cleveland, Ohio, USA), 25 mg kg⁻ or dexamethasone, 4 mg kg⁻ was injected i.v 60 min prior to the induction of hypertension. Haloperidol, 1 mg kg⁻¹ was given i.v 30 min befor the hypertensive drug. For the number of rats in the various groups see Table I. Before the blood pressure increase was induced, 2 ml of a 2 %

Table II. ^{125}I-HSA content in the brain 3 min after the administration of bicuculline (1.2 mg kg^{-1}) in controls and in rats pretreated with SITS, dexamethasone or haloperiodol. The tracer content is expressed as 100 (counts min^{-1} mg^{-1} brain tissue)/(counts min^{-1} mg^{-1} blood). Mean values ± S.E. (n 6 in all groups).

Experimental group	Cerebral cortex	Thalamus	Cerebellum
Control	0.18 ± 0.03	0.94 ± 0.17	0.29 ± 0.03
SITS (25 mg kg^{-1})	0.08 ± 0.01	0.22 ± 0.01	0.09 ± 0.02
Dexamethasone (4 mg kg^{-1})	0.11 ± 0.01	0.50 ± 0.13	0.2[illegible] ± 0.05
Haloperidol (1 mg kg^{-1})	0.15 ± 0.02	0.71 ± 0.08	0.23 ± 0.02

SITS 4 acetamido-4-isothiocyano-stilbene 2,2 -disulfonic acid disodium.
*$p<0.05$, ** p 0.01 for differences from control rats.

solution of Evans blue in saline kg^{-1} (which in vivo binds to serum albumin) and 100 μCi kg^{-1} of ^{125}I-HSA (human serum albumin) were injected 3 min after the administration of adrenaline, 20 μg kg^{-1} or bicuculline, 1.2 mg kg^{-1} the brain was perfused with saline in situ for 1 min to rinse the cerebral vessels from blood. Predetermined parts were subjected to scintillation counting and the extravasation of ^{125}I-HSA was expressed as 100 (counts min^{-1} mg^{-1} brain tissue)/(counts min^{-1} mg^{-1} blood). The blood sample was taken immediately before starting of the perfusion through the heart.
Statistical differences were evaluated with Wilcoxon rank sum test.

Results

MAP before and after the pressure increase and PaCO values immediately prior to the administration of bicuculline or adrenaline are given in Table I. Macroscopically Evans blue-albumin extravasation was less in the SITS treated groups and ^{125}I-HSA leakage was significantly less in all areas of the brain in rats given SITS (Table II and III). This was particularly evident in rats given adrenaline in spite of higher $PaCO_2$ in the SITS treated group. Thus 7 out of 8 treated rats showed no or little extravasation and the IHSA ratio was 0.03–0.08. (The tracer content in control brains from rats not subjected to hypertension is 0.–0.4 with the present technique of perfusion, see Carlsson and Johansson 1978). However as can be seen from the mean value and large standard error (Table III) no protective effect was seen in one rat in the SITS-treated group. Compared to controls there was less extravasation also after pretreatment with dexamethasone but the decrease was significant only for the cerebral cortex. (Table II.) Haloperiodol had no protective effect on protein leakage in the present experimental situation.

Table III. ^{125}I-HSA content in the brain 3 min after an injection of adrenaline (20 μ kg^{-1}) in controls and in rats pretreated with SITS. The tracer content is expressed as 100 (counts min^{-1} mg^{-1} brain tissue) (counts min^{-1} mg^{-1} blood). Mean values ± S.E. (= 8 in both groups).

Experimental group	Telencephalon	Diencephalon, mesencephalon	Pons, medulla oblongata	Cerebellum
Controls	0.47 _ 0.13	0.36 ± 0.10	0.33 ± 0.07	1.16 + 0.50
SITS (25 mg kg)	0.18 _ 0.11	0.10 ± 0.03	0.10 ± 0.02	0.23 ± 0.13

SITS 4 acetamido-4-isothiocyano-stilbene-2,2 -disulfonic acid disodium.
p [illegible]

[illegible]

Discussion

The results obtained in the present study demonstrate that SITS reduces the protein extravasation occurring in the brain after an abrupt increase of the blood pressure in rats. Theoretically SITS could either medrate a change in the cerebrovascular tone or could have a direct effect on the endothelial cell membrane. Although the first possibility cannot be excluded until hemodynamic studies have been performed an action on the cell membrane seems more likely

In the present experimental models the extravasated proteins are probably mainly transported across the endothelial cells by pinocytosis (ref see Introduction) Very few vesicles are observed in cerebral vessels under normal conditions (Reese and Karnovsky 1967) The wall distension induced by a rise in blood pressure seems to activate the endothelial cell membrane to form pinocytotic vesicles. It should be stressed that the formation and transendothelial transport of such vesicles are transitory phenomena in the present experimental models. When the blood pressure returns to normal levels the protein leakage ceases within 10–15 min (Petito Schaefer and Plum 1977 Johansson and Linder 1978). Pinocytosis is an active process requiring activation of adenyl cyclase (Masur *et al* 1971 Joo Rakonczay and Wollemann 1975) It has been demonstrated in another system that SITS prevents the activation of adenyl cyclase induced by treatment of intestinal cells as well as of lymfocytes with cholera toxin (Lönnroth *et al* in preparation) Furthermore, SITS inhibits the ATP-evoked epinephrine release from isolated chromaffin granules as well as the serotonin secretion from human platelets, induced by stimulation with thrombin or the calcium ionophor A23187 (Hoffman *et al* 1976, Pollard *et al* 1977), both processes being dependent upon adenyl cyclase activation.

Obviously further studies are needed to evaluate the mechanisms behind the protective effect of SITS on pressure-induced BBB dysfunction Work is in progress ot study if SITS has any prophylactic effect on other types of BBB dysfunctions.

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Acta physiol scand. 1979 105. 518–520

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Paresthesiae. Abnormal impulse generation in sensory nerve fibres in man

By

H Erik Torebjörk, José L. Ochoa and Frances V McCann

The nature of the mechanisms underlying positive sensory symptoms, like spontaneous pai and paresthesiae, occurring in the setting of peripheral nerve dysfunction has remaine speculative for powerful reasons. Indeed prerequisites to any investigation of that questio include the ability to record, in alert human subjects, peripheral nerve signals that can b correlated with abnormal sensory experience and further the ability to analyse the patter of activity in single afferent fibres of various types in relation to the spectrum of perverte sensations Means to fulfill these requirements are now available. Using the microneurc graphic technique of Vallbo and Hagbarth (1968) we have recorded peripheral nerve signal from single afferent fibres in awake subjects experiencing paresthesiae. Possible ethic reservations were circumvented by recording signals from our own peripheral nerves.

Post-ischemic paresthesiae were induced after release of a sphygmomanometer cuff th had remained inflated well above systolic blood pressure round the upper arm or the fore arm for 15 to 40 min (Lewis *et al* 1931 Zotterman 1933 Kugelberg 1944 Weddell an Sinclair 1947 Gilliatt and Wilson 1954 Nathan 1958) Neural signals were recorded wit semi-microelectrodes inserted into sensory nerve fascicles of the median or the ulnar nerve proximal or distal to the site of the cuff Details concerning signal recording, amplificatior storage, and processing have been given elsewhere (Hagbarth *et al* 1970) Fifteen experi ments were performed on three healthy volunteers, and were not followed by complication of any kind.

Within 20 s of decompression and reestablishment of circulation to the cold numb hand sensation of warmth rapidly leading to hot was perceived in the whole blushing hand Ap proximately 40 to 60 s following release, high speed "buzzing" sensations of increasin intensity were referred predominantly to finger tips. Intensity peaked by *90 s strikin* spontaneous electrical discharges could be recorded during this period Fig. 1 A shows th beginning of a characteristic high frequency discharge of relatively long duration (*up to 7 s*) Impulse frequency *slowly* decayed from an initial maximum as high as 240 imp/s. Recurrin "buzzing" sensations and periods of sustained high frequency discharges were commonplac during this most intense stage of paresthesiae

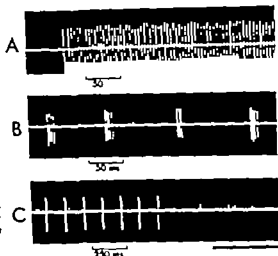


Fig. 1. Single unit impulses recorded in sensory nerve fascicles of the median nerve [illegible] show [illegible] during periods of post-ischemic paresthesiae after release of cuff around the [illegible]. B and C represent activity in the same unit, [illegible] different from the unit in A. A. Abrupt beginning of high frequency discharge, lasting for several seconds, [illegible] subject reported sensations of prolonged "buzzing". B. Brief bursts of high frequency discharges repeated in [illegible] rhythmic fashion. Subject felt intermittent "tingling". C. Cessation of repeated double discharges was closely followed by subject's report of disappearance of faint "tingling" (bar).

As "buzzing" progressively diminished, distinct sensations described as intermittent "tingling" and short but painless "pins and needles" became the dominant feature between 90 and 120 s. Such sensations, which were also predominantly referred to finger tips, eventually substituted "buzzing" altogether. Pins and needles waned by the 4th minute, and then tingling slowly decayed, to disappear after 10–20 min. Towards the end of this decrescendo period, it was often possible to identify discrete points of referral in the skin where the other, more chaotic, intermittent paresthesiae, could be perceived individually in a rhythmic fashion. The neural activity recorded during intermittent paresthesia was dominated by brief bursts of high frequency discharges or by individual discharges sometimes repeated at various rhythms (Fig. 1 B). With time, the number of spikes per burst diminished and the interval between bursts increased, as the tingling sensation got fainter. In one occasion the subject deprived from audiovisual feedback reported disappearance of tingling referred to an identified field in the finger tip when the unit ceased to fire (Fig. 1 C).

These findings, based upon the application to man of techniques for single unit recording, confirm the prediction that paresthesiae from peripheral nerve dysfunction signify abnormal impulse generation in the sensory unit. Our studies indicate that recognizable changes in perception of paresthesiae (sustained "buzzing" versus intermittent "tingling") can be the consequence of modulation of impulse frequency and duration and rhythm of the discharges. Although the contribution to paresthesiae from different fibre types has not been investigated, the sustained, very high frequency of discharge recorded in some fibres endorses participation of myelinated fibres.

Studies are now in progress to help define the sites of abnormal impulse generation within the sensory unit, and to explore spontaneous activity in the spectrum of nerve fibre types during paresthesiae and neuritic pain.

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Are there sensory neurons in the mucosa of the mammalian gut?

By

B. Newson, H. Ahlman, A. Dahlström, T K. Das Gupta and L. M Nyhus

The innervation of the gastrointestinal (G I.) canal in mammals classically includes two plexa of ganglion cells, the submucous plexus and the myenteric plexus. Some nerve cells of these two plexa are probably of a sensory type (type II according to Dogiel 1896) and may send processes to the submucous–subglandular layer of the G.I. canal. However the occurrence of additional small nerve cells with elongated processes in the subglandular layer of the cow and pig stomach has been described (Vau 1932). During our studies on the morphological connection between nerves and epithelial cells of the small intestine of rat, we observed similar elongated nerve cells just beneath the crypt epithelium.

Male rats 150 to 200 g were decapitated. Small mucosal specimens were prepared from the ileum. The specimens were fixed in 4% buffered glutaraldehyde, postfixed in 2% OsO_4, dehydrated, and embedded in Araldite Ultrathin sections (600 Å) stained with lead citrate and uranyl acetate were observed under a transmission electron microscope (EMU 4 RCA)

In sections from the ileum, small, elongated cells with a light nucleus and floccular rough endoplasmic reticulum (rER) were sometimes observed just beneath the epithelium of the crypts Their elongated processes were often separated from the crypt cells only by a basal lamina (Fig. 1). Membrane thickenings were occasionally seen on opposite sides of the separating lamina (Fig. 1 b). The processes, extending in parallel with the epithelium lining, mostly contained patches of rER, mitochondria, vesicles of varying sizes and electron-densities, and occasionally microtubules. Other processes seemed devoid of rER but contained smooth ER tubules. This type of cell has the morphological characteristics of a nerve cell, and the extension of rER-containing processes (probably dendrites) may suggest that the neuron is of a sensory type. Support of the view that these cells are neurons is obtained from light microscopic immunohistochemical studies using Thy-1 antigens. The Thy-1 antigen has been shown to be present in large amounts in cell membranes of the thymus and neuronal elements in the rat, and appears to be specific for these two tissues in the adult rat (cf. Barclay and Hydén 1978). In the ileum Thy-1 positive cells were observed not only in the two nervous plexa of the gut wall, but also in elongated cells located just beneath the

Key words: Rat ileal mucosa, sensory neurons.

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epithelial lining of the crypt glands (H.-A. Hansson, pers. communication) i.e. the same localization as the cells of the present study.

The presence of subepithelial sensory neurons in the adult mammalian gut has not been demonstrated earlier but the occurrence of subglandular small ganglion cells in the stomach of cow and pig has been described (Vas 1932). This author described the cells as small—to medium-sized, elongated, with a light, eccentric nucleus. The neuronlike cells located near the crypt epithelium in the rat small intestine appear to have similar characteristics. There is physiological evidence for a sensory nervous input to the small intestine (cf. Biber et al. 1971) and the neuronlike cells observed in this study may possibly contribute part of such sensory innervation of the gut.

Supported by the Veterans Administration West Side Hospital, the C. T. Burgheim Foundation and the Swedish Medical Research Council (17X 5220, 14X 2207, 04P-4173).

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Fig. 1 Montage of electronmicrographs of a neuronlike cell (arrows) the ileal mucosa. The cell has a light nucleus (N), two smaller processes (top) nd one large process (bottom). The cytoplasm of the large process contains rough endoplasmic reticulum, mitochondria and vesicles of ying appearance. The elongated cell is located just underneath the epithelium. Bar represents 500 nm. *Inlet* Detail from a similar neuronlike cell demonstrates multiple nexus areas between the cell and adjacent i testinocyt (arrows). Bar represents 400 nm.

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Fig. 1 Montage of electronmicrographs of a neuronlike cell (arrows) in the ileal mucosa. The cell has a light nucleus (N), two smaller processes (top) and one large process (bottom). The cytoplasm of the large process contains rough endoplasmic reticulum, mitochondria and vesicles of varying appearance. The elongated cell is located just underneath the epithelium. Bar represents 500 nm. *Inlet:* Detail from a similar neuronlike cell demonstrates multiple nexus areas between the cell and an adjacent [illegible] (arrows). Bar represents 400 nm.

Table I. Effect of lipid peroxidation on fatty acids and phospholipids in brain cortical tissue. Paired samples of cortex homogenates were incubated with ferrous sulfate and ascorbic acid either in an aerobic (5 [illegible] O_2) or in an anaerobic (100% N_2) atmosphere (for details see text). The values (μmol g^{-1} cortex) are means ± S.E. of 6 expts. Statistical differences were evaluated with Student's t-test for paired samples.

	5 [illegible] O_2	100% N_2	Probability (p)
Total fatty acids	113 ± 4	122 ± 3	$p < 0.01$
20:4[a]	10.8 ± 0.4	12.3 ± 0.4	$p < 0.01$
[illegible]:6	14.8 ± 0.7	18.7 ± 0.4	$p < 0.001$
Ethanolamine phosphoglyceride	22.5 ± 0.6	26.5 ± 0.5	$p < 0.001$
Choline phosphoglyceride	25.1 ± 0.7	25.0 ± 0.3	n.s.

[a] Number of carbon atoms: number of double bonds.

Lipids were extracted with chloroform methanol (1 1) and separated by silica gel thin-layer chromatography. Phospholipids were quantitated as P (total phosphorous) and fatty acids were analyzed by gas chromatography after conversion to methyl esters (Åkesson *et al.* 1970).

There was an appreciable amount of TBA-reactive material formed (0.638 ± 0.062 μmol (malondialdehyde) g^{-1} cortex) in samples incubated with oxygen, indicating peroxidation. In samples incubated with 100% N the amount of TBA-reactive material was negligible (0.008 ± 0.002 μmol (malondialdehyde) g^{-1} cortex).

As the table shows, there were clear differences in tissue levels of phospholipids and fatty acids between the O_2-equilibrated (and peroxidized) sample and the sample tonometered with N_2. When the values were compared to zero time controls (not shown) it was apparent that phospholipid breakdown due to anaerobic phospholipase activity was undetectable. In contrast, aerobic incubation with its accompanying lipid peroxidation was followed by a decrease in ethanolamine phosphoglyceride concentration, with no change in choline phosphoglyceride, and by loss of arachidonic and docosahexaenoic acids. The latter results were thus similar to those previously reported to occur during lipid peroxidation in microsomal preparations from liver tissue (Recknagel and Ghoshal 1966, May and McCay 1968).

In conclusion, the present results demonstrate that selective changes in brain tissue phospholipids and fatty acids occur during peroxidation *in vitro* It should thus be feasible to estimate the presence of lipid peroxidation *in vivo* by analyzing phospholipids and fatty acids in either the whole tissue, or in one of its subcellular fractions.

The study was supported by grants from the Swedish Medical Research Council (No. 14X 263 and 03P [illegible]), from the Knut and Alice Wallenberg Foundation and from the U.S. Public Health Service (No [illegible]) from N I H. The authors are grateful to Lena Sjöberg for excellent technical assistance.

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Lipid peroxidation in brain tissue *in vitro* Effects on phospholipids and fatty acids

By

EVA WESTERBERG BJÖRN ÅKESSON STIG REHNCRONA, DAVID S SMITH and BO K. SIESJÖ

It is currently debated whether or not lipid peroxidation is one of the mechanisms wherel cell damage occurs in a variety of adverse conditions, *e.g* those caused by radiation, hyp oxia, and carbon tetrachloride intoxication (see review by Slater 1972) When brain tiss are incubated *in vitro* in the presence of oxygen and suitable free radical initiators, a preciable amounts of lipid peroxides are formed (for literature, see Barber and Bernhei 1967) Recently it was hypothesized that the irreversible neuronal damage that occurs fo lowing incomplete cerebral ischemia is due to dislocation of free radicals and a resulti peroxidative damage to intracellular membranes (Demopoulos *et al* 1977).

It has been difficult to prove that lipid peroxidation occurs *in vivo* This is mainly due the fact that the major tests used for estimating peroxidation *in vitro* *e.g* the thiobarbitur acid (TBA) method for measuring malondialdehyde and related peroxide cleavage product is not applicable to *in vivo* situations A promising approach is the estimation of *in vi* changes in tissue phospholipids and fatty acids since *in vitro* studies have shown that lip peroxidation preferentially effects ethanolamine phosphoglyceride and the polyunsaturate fatty acids, arachidonic acid and docosahexaenoic acid (Recknagel and Ghoshal 1966, M and McCay 1968) However if applied to brain tissue and to changes occurring in ischem this approach is only valid if it can be shown that changes in fatty acids due to peroxidatio are clearly different from those caused by anaerobic mechanisms. In order to test this, w incubated cerebral homogenates in the presence of free radical initiators, with and withou oxygen, and determined changes in phospholipids and fatty acids.

Male Wistar rats were decapitated i to liquid nitrogen. The brains were chiselled out and cortical tiss (400 mg) was dissected at −22°C. The tissue was the homogenized at 0°C in 4 ml of phosphate buff (0.05 M) containing Na in a final concentration of 0.015 M and K 0.145 M, (pH 7.0), prequilibrate with N_2. The homogenates were split int paired samples and tonometered at 37°C for 45 min in the presenc of ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM), either with 5% O_2 or with 100% N_2. At the en of incubation, aliquots were taken for determination of TBA-reactive material (Slater and Sawyer 1971

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Lipid peroxidation in brain tissue *in vitro*
Antioxidant effects of barbiturates

By

David S. Smith, Stig Rehncrona, Eva Westerberg, Björn Akesson and Bo K. Siesjö

Current information suggests that various barbiturates can ameliorate the adverse effects of complete or incomplete cerebral ischemia on cell viability and neurological function (for literature, see Michenfelder *et al* 1976). The mechanism(s) of protection have not been defined. Available results do not favour the view that the decrease in cerebral metabolic rate, which accompanies barbiturate anesthesia, can alone account for the protection (Nordström *et al* 1978). It was recently suggested that free radical formation occurs during incomplete ischemia induced by middle cerebral artery occlusion in the cat, indicating that peroxidative damage to intracellular membranes is an important mechanism of cell injury. Furthermore, since methohexital inhibited the production of thiobarbituric acid (TBA) reactive material in liposomes irradiated with UV light, it was suggested that barbiturates protect brain tissue against ischemia by acting as free radical scavengers (Demopoulos *et al* 1977, Flamm *et al.* 1978). In order to test this hypothesis we devised a system for inducing lipid peroxidation in brain tissue *in vitro* and studied the antioxidant ("scavenging") effects of several barbiturates, comparing their efficiency with that of an established free radical scavenger (promethazine).

Following decapitation of male Wistar rats, their heads were immediately frozen in liquid nitrogen, and the brains chiselled out. Cortical tissue (400 mg) was dissected at −22°C and homogenized anaerobically at 0°C in 4 ml of 0.05 M phosphate buffer (pH 7.0) which had a final concentration of 0.145 M potassium and 0.015 M sodium. The brain homogenate was split into duplicate samples which were incubated for 1 h at 37°C in tonometers equilibrated with 5 O in N. Lipid peroxidation was enhanced by adding ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM) to the homogenates. Samples (0.5 ml) were obtained after 0, 30, 45 and 60 min of incubation. Lipid peroxidation was estimated by analyzing TBA reactive material using malondialdehyde as standard. In separate experiments, samples were obtained for analysis of phospholipid-bound fatty acids (see preceding article by Westerberg *et al.* 1979). Various compounds were tested for their ability to inhibit malondialdehyde production. These were added to one of the homogenates just prior to the addition of

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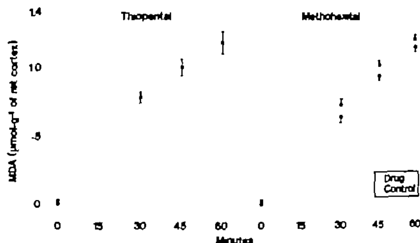


Fig. 1. Effects of thiopental or methohexital on Fe^{2+} induced lipid peroxidation in brain homogenate. Samples for analysis of malondialdehyde (MDA) were obtained after 0, 30, 45, and 60 min of incubation with 5% oxygen in nitrogen. The samples were paired, with each brain serving as its own control. The symbols represent the mean ± S.E. from 4 rats. Except for the zero time sample the groups are significantly different ($P < 0.05$) from their controls.

ascorbic acid and iron. Statistical analysis of the results was done using Student's t-test for paired samples.

When the brain homogenates were incubated without iron and ascorbic acid, moderate amounts (0.3 μmol·g⁻¹) of malondialdehyde (MDA) accumulated during 1 h of incubation. In the presence of these free radical initiators, MDA production usually exceeded 1 μmol·g⁻¹. Thiopental (1 mM) produced 96% inhibition of MDA formation, *i.e.* the values were less than those obtained in the absence of added initiators. However, promethazine was even more efficient since it gave a comparable inhibition at a concentration of 0.1 mM. Methohexital (1 mM) produced some inhibition but its degree (8%) was much less than that produced by thiopental (Fig. 1). Phenobarbital (1 mM) or pentobarbital (1 mM) had no effect on MDA production.

Analysis of fatty acids showed that thiopental not only inhibited the production of MDA but it also inhibited the associated lipid oxidations. As described in the preceding article (Westerberg *et al.* 1979) there was an oxygen-induced loss in the sum of arachidonic and docosahexaenoic acid (18%) when the homogenates were incubated with iron and ascorbic acid. Thiopental completely prevented this change in fatty acid composition.

The present results give little support to the hypothesis that barbiturates protect the brain in ischemia by acting as free radical scavengers. Thus, whereas protection *in vivo* has been reported with thiopental, methohexital, pentobarbital and phenobarbital, only thiopental was efficient as a free radical scavenger when tested on our brain homogenate model. However, it is often difficult to extrapolate *in vitro* effects of drugs to an *in vivo* situation, and it seems necessary to study the protective effect, if any, of promethazine and related compounds before commenting further on the free radical hypothesis.

The study was supported by grants from the Swedish Medical Research Council (No. 14X 263 and 03P 4931), from the Knut and Alice Wallenberg Foundation and from the U.S. Public Health Service (No. R01NS07838) via N.I.H. The authors are grateful to Lena Sjöberg for excellent technical assistance.

Abstracts from Meeting of the Scandinavian Physiological Society in Odense 3–4 November 1978

COMMUNICATIONS

C 1

Fatty Acid Mobilization Limited by Adipose Tissue Blood Flow During Exercise*

By J. BÜLOW and J. MADSEN *Institute of Medical Physiology C University of Copenhagen, Denmark*

We have previously demonstrated an increase in adipose tissue blood flow (ATBF) during exercise (Bülow and Madsen 1976). The purpose of the present study was to assess the free fatty acid (FFA) carrying capacity of plasma albumin under conditions where ATBF limited FFA mobilization in order to evaluate whether ATBF may be limiting for FFA mobilization during exercise

Denervated canine subcutaneous inguinal fat was perfused at a constant rate Lipolysis was stimulated by addition to the blood of isoprenaline 3×10^{-4} M and theophyllamine 1×10^{-4} M In venous plasma from stimulated adipose tissue the $C_{FFA}/C_{albumin}$ ratio was 4.5 (mol/mol) at control ATBF (3.8 ml/100 g/min) If ATBF was reduced to 70% of the control value FFA release decreased to 25% A reduction of ATBF to 6% caused a reduction of FFA release to 10% control value. In these two situations mean $C_{FFA}/C_{albumin}$ were 6.8 and 7.4 respectively Values >10 were never observed

Canine and human plasma (pH 7 4) were equilibrated with heptane solutions of palmitic acid at increasing concentrations. 4–6 moles of FFA were bound to 1 mol of albumin at very low concentrations in the heptane phase The following moles required increasingly higher concentrations in the heptane phase No consistant difference between the binding capacities of human and dog plasma was observed

8 volunteers worked at a bicycle ergometer for 4 hours at 50% of their V_{O_2} max During work their adipose tissue blood flow increased to a mean value of 10 ml/100 g/min. Average V_{O_2} and V_{CO_2} in the 4th hour were 1.59 and 1.27 l/min. Assuming a protein catabolism of 0 1 g/min, lipid metabolism can be calculated to 0 50 g/min. Taking this to be tripalmitate 1 86 mmol FFA is mobilized and metabolized per minute Assuming 10% of the body weight to be adipose tissue, 26.6 μmol FFA are mobilized per 100 g adipose tissue per minute They are transported by 10 0.55 ml plasma that has a FFA concentration of 4 7 μmol/ml before entering the tissue. With a plasma albumin concentration of 0 7 μmol/ml, the $C_{FFA}/C_{albumin}$ in adipose tissue venous blood should be about 13–14 Thus, it appears very likely that ATBF limits FFA mobilization during prolonged heavy exercise Calculations based on reported figures for C_{FFA} and V_{CO_2} during prolonged fasting suggest that ATBF may also be critical for FFA mobilization in this situation

Reference

Bülow, J. and J. Madsen *Pflügers Arch* 1976 363 231–234

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Oslo. Parts of the operating technique and data processing have been described elsewhere (Eskilsen 1975 Wille 1977). The instrument ("UNIDOP") discriminates between forward and backward blood velocities by "heterodyne detection. The emitted sound beam can be either pulsed or continuous. In the pulsed mode the instrument is only sensitive to blood velocities occurring in a certain adjustable distance from the probe. Two ultrasound frequencies are available, 1.5 MHz, suitable for measurements on large deep vessels and in the heart, and 6 MHz, for measurements on superficial vessels (less than 2.5 cm from the body surface). In the present investigation 6 MHz pulsed sound was used. Velocity spectra were calculated from the doppler signal on-line in a NORD computer system. The computer also calculated the instantaneous mean velocity across the vessel lumen and the time average of this mean velocity for each successive cardiac cycle. The instantaneous mean velocity is proportional to the instantaneous blood flow through the vessel as long as certain measurement conditions are fulfilled. The proportionality constant is equal to the cross-sectional area of the vessel and thus different for different vessels. At present either knowledge about this area or calibration against another method (e.g. plethysmograph) is needed to convert the velocity measurements to flow. However if the relative changes in blood flow with time are interesting in itself no calibration is necessary.

To illustrate the possible uses of this instrument we have reinvestigated the variability of the blood flow in the human hand at different environmental temperatures. This problem was originally studied by Burton (1939) with a finger plethysmograph. Our investigation confirms Burton's findings of a tremendous range of blood flow values in the fingers and of marked and rapid fluctuations in this flow in resting subjects. It does not, however, confirm Burton's description of the fluctuations as short episodes of vasoconstrictions occurring with different frequencies on basically dilatated vessels.

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C 4

Determination of the Interendothelial Cleft Length in the Frog Mesenteric Capillary

By M. BUNDGAARD, J. FRØKJÆR JENSEN and C. CRONE. *Institute of Medical Physiology, Dept. A, The Panum Institute, Copenhagen, Denmark.*

The interendothelial cleft is generally thought to be the main pathway for the transcapillary passage of small hydrophilic solutes. The total length of the cleft per unit surface area in the

C 2

Post—exercise Rise in Plasma Concentration of Free Fatty Acids (FFA) and Adipose Tissue Blood Flow

By TERJE LARSEN HARALD VIK MO KJELL MYHRE and OLE D MJØS *Department of Physiology Institute of Medical Biology University of Tromsø Norway*

Male Wistar rats were subjected to treadmill exercise for 30 minutes Arterial concentrations of FFA glycerol and lactate were measured before during and after exercise The arterial concentrations of both glycerol and lactate rose markedly during exercise and fell after its termination The plasma concentration of FFA however increased only slightly during the exercise period while a sharp increase in plasma FFA was noted upon termination of exercise this amounted to a maximum of 1439±89 μmol/l (mean ± S E M) after 6 minutes of recovery Thereafter plasma FFA fell In order to study whether the post-exercise rise in plasma FFA concentration could be related to changes in adipose tissue blood flow radioactively labelled microspheres were injected in the aorta under the various experimental conditions This made it possible to estimate the fraction of cardiac output going to adipose tissue (FCO) FCO was reduced early in the exercise period compared with resting values but increased up to resting values at the end of exercise. However 3 minutes after termination of exercise FCO was markedly increased reaching values 2–4 times greater than the values at the end of exercise We estimated that a simultaneous rise in adipose tissue blood flow had occurred It is possible that during exercise FFA concentration within adipose tissue is increased The peak in plasma FFA concentration observed during the early recovery period might therefore be due to FFA release from adipose tissue as a consequence of increased adipose tissue blood flow in the same period

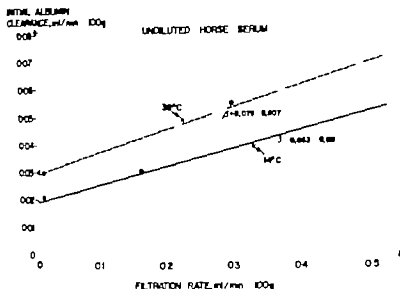
C 3

Rapid Changes in Arterial Blood Flow in Humans Measured Non-invasively by an Ultrasonic Doppler Velocimeter

By MARIANNE THORESEN and LARS WALLØE *Institute of Physiology University of Oslo Norway*

In the early 1960-ies Rushmer Baker and collaborators developed an ultrasonic doppler instrument which could measure blood velocities through the skin (e g Rushmer et al 1966) Theoretically the doppler signal from such an instrument should contain information about all velocities present in the vessel and about the relative amount of blood flowing with each velocity However the technical problems involved have been substantial and the instruments that are available commercially and used clinically today operate more like "flow detectors" than as "velocity meters"

Many attempts have been made to improve the original instruments (see Woodcock 1975 for references) The instrument used in the present investigation is the result of such an attempt and was developed in collaboration between several university departments in



0.8 ml/min × 100 g, increasing linearly with filtration rate to some 0.07 ml/min × 100 g at 0.5 ml/min 100 g of filtration. During cooling from 36°C to 14°C both CFC and initial albumin clearance at isogravimetry decreased some 40% in due proportion to the increased viscosity of the fluid (see Figure). Increases of the colloid osmotic pressure of the perfusate correspondingly increased both the isogravimetric capillary pressure and initial albumin clearance during isogravimetry. Initial albumin clearance at maximal vasodilatation seemed to follow the relation

$$CL = PS + \sigma(1-\sigma)\,CFC\,\Delta TC + F\,(1-\sigma)$$

where CL is the albumin clearance (ml/min × 100 g), PS the capillary diffusion capacity for albumin (ml/min × 100 g), F net volume flow across the microvascular wall, CFC capillary filtration coefficient (ml/min × mmHg 100 g), ΔTC the colloid osmotic pressure difference over large pores (mmHg) and σ the mean osmotic reflection coefficient for albumin. Experimentally (1 σ (β see Figure) averaged 0.072, CFC 0.036, PS 0.009 and at a plasma colloid osmotic pressure of 20.5 mmHg CL was estimated to 0.029 during isogravimetry.

It is concluded that during isogravimetry at ordinary capillary pressures the transmicrovascular albumin passage is to about 70 per cent due to filtration whereas only some 30 per cent of transport takes place by diffusion, both events presumably via 'large pores'. There was no evidence that transendothelial vesicular transport should to any significant extent contribute to the passage of albumin from vessel to tissue.

mesenteric capillary is unknown Using stereological methods to analyse electronmicroscopic pictures of transverse sections of 49 capillaries from the frog mesentery a total cleft length per μm^2 of 2400 Å±975 (S D) was found It is possible to evaluate this figure and conclude that a large part of the interendothelial cleft must be open in order to comply with determinations of solute permeability

Crone *et al* (1978) using a pore model calculated an equivalent pore radius r of 110 Å in the frog mesenteric capillary This corresponds to an average cleft width w of 134 Å if the passage occurs through a slit-like structure rather than through a pore ($w = r\sqrt{3}/2$) Knowing the cleft width the open cleft length L_c can be obtained as follows $P = (D_k \cdot w \cdot L)/\Delta x$ where P_k is the potassium permeability (67×10^{-6} cm sec) D_k is the free diffusion coefficient of potassium at 20°C ($1\ 8 \times 10^{-5}$ cm² sec) and Δx the average path length in the capillary wall (5×10^{-6} cm) Solution for L gives L = 1 390 Å per μm^2 capillary surface area When this figure is expressed relative to the total morphologically determined cleft length it is found that about 58 % of the cleft must be open

Lassen and Trap-Jensen (1970) from theoretical calculations concluded that in the less permeable muscle capillary 18 % of the cleft was open Perl (1971) from somewhat different assumptions arrived at a figure of 3 3 % while Casley Smith *et al* (1975) found 4.8 % for the muscle capillary

It is likely that the junctional dimensions in various capillaries is rather constant and that variations in permeability between organs are explained by variations in the fractional open cleft lengths

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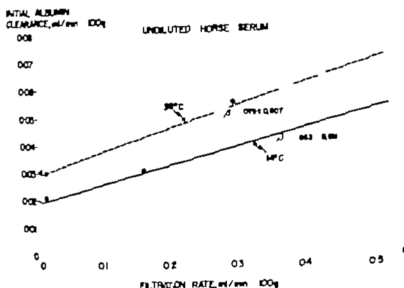
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C 5

Transcapillary Passage of Albumin Effects of Tissue Cooling and of Increases in Filtration and Plasma Colloid Osmotic Pressure

By B RIPPE A KAMIYA and B FOLKOW *Department of Physiology University of Göteborg Sweden*

In order to study the mechanisms behind the transfer of macromolecules across microvascular walls 'initial' clearance of radiolabelled serum albumin was measured in the perfused maximally vasodilated muscle vascular bed of rat hindquarters during tissue cooling during increases in filtration and during changes in serum colloid osmotic pressure Albumin clearance during ordinary serum perfusion at isogravimetry amounted to



1.8 ml/min × 100 g, increasing linearly with filtration rate to some 0 07 ml/min × 100 g at 0.5 ml/min 100 g of filtration During cooling from 36°C to 14°C both CFC and initial albumin clearance at isogravimetry decreased some 40 % in due proportion to the increased viscosity of the fluid (see Figure) Increases of the colloid osmotic pressure of the perfusate correspondingly increased both the isogravimetric capillary pressure and initial albumin clearance during isogravimetry Initial albumin clearance at maximal vasodilatation seemed to follow the relation

$$CL = PS + \sigma(1-\sigma)\,CFC\,\Delta TC + F_v(1-\sigma)$$

where CL is the albumin clearance (ml/min × 100 g) PS the capillary diffusion capacity for albumin (ml/min × 100 g) F net volume flow across the microvascular wall CFC capillary filtration coefficient (ml/min mmHg × 100 g) ΔTC the colloid osmotic pressure difference over large pores (mmHg) and σ the mean osmotic reflection coefficient for albumin. Experimentally (1−σ (β see Figure) averaged 0 072, CFC 0 036 PS 0 009 and at a plasma colloid osmotic pressure of 20 5 mmHg CL was estimated to 0 029 during isogravimetry

It is concluded that during isogravimetry at ordinary capillary pressures the transmicrovascular albumin passage is to about 70 per cent due to filtration, whereas only some 30 per cent of transport takes place by diffusion, both events presumably via large pores. There was no evidence that transendothelial vesicular transport should to any significant extent contribute to the passage of albumin from vessel to tissue

mesenteric capillary is unknown Using stereological methods to analyse electronmicroscopic pictures of transverse sections of 49 capillaries from the frog mesentery a total cleft length per μm^2 of 2400 Å±975 (S D) was found. It is possible to evaluate this figure and conclude that a large part of the interendothelial cleft must be open in order to comply with determinations of solute permeability

Crone *et al* (1978) using a pore model calculated an equivalent pore radius r of 110 Å in the frog mesenteric capillary This corresponds to an average cleft width w of 134 Å if the passage occurs through a slit like structure rather than through a pore ($w=r/3/2$) Knowing the cleft width the open cleft length L, can be obtained as follows $P_k=(D_k\ w\ L)/\Delta x$ where P_K is the potassium permeability (67×10^{-6} cm sec $^{-1}$) D_K is the free diffusion coefficient of potassium at 20°C (1 8×10^{-5} cm^2 sec) and Δx the average path length in the capillary wall (5×10^{-5} cm) Solution for L gives L=1 390 Å per μm^2 capillary surface area When this figure is expressed relative to the total morphologically determined cleft length it is found that about 58 % of the cleft must be open

Lassen and Trap-Jensen (1970) from theoretical calculations concluded that in the less permeable muscle capillary 18 % of the cleft was open Perl (1971) from somewhat different assumptions arrived at a figure of 3 3 % while Casley Smith *et al* (1975) found 4 8 % for the muscle capillary

It is likely that the junctional dimensions in various capillaries is rather constant and that variations in permeability between organs are explained by variations in the fractional open cleft lengths

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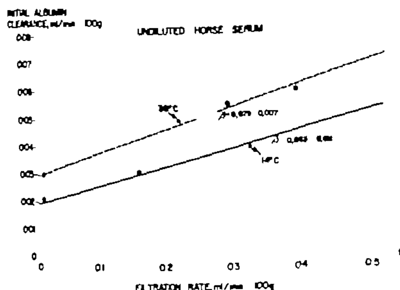
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C 5

Transcapillary Passage of Albumin Effects of Tissue Cooling and of Increases in Filtration and Plasma Colloid Osmotic Pressure

By B RIPPE A KAMIYA and B FOLKOW *Department of Physiology University of Göteborg Sweden*

In order to study the mechanisms behind the transfer of macromolecules across microvascular walls initial' clearance of radiolabelled serum albumin was measured in the perfused maximally vasodilated muscle vascular bed of rat hindquarters during tissue cooling during increases in filtration and during changes in serum colloid osmotic pressure Albumin clearance during ordinary serum perfusion at isogravimetry amounted to



10 ml/min × 100 g increasing linearly with filtration rate to some 0 07 ml/min × 100 g at 0.5 ml/min 100 g of filtration During cooling from 36°C to 14°C both CFC and initial albumin clearance at isogravimetry decreased some 40% in due proportion to the increased viscosity of the fluid (see Figure) Increases of the colloid osmotic pressure of the perfusate correspondingly increased both the isogravimetric capillary pressure and 'initial' albumin clearance during isogravimetry Initial albumin clearance at maximal vasodilatation seemed to follow the relation

$$CL = PS + \sigma(1-\sigma)\ CFC\ \Delta TC + F\ (1-\sigma)$$

where CL is the albumin clearance (ml/min × 100 g) PS the capillary diffusion capacity for albumin (ml/min 100 g) F net volume flow across the microvascular wall, CFC capillary filtration coefficient (ml/min mmHg × 100 g) ΔTC the colloid osmotic pressure difference over large pores (mmHg) and σ the mean osmotic reflection coefficient for albumin Experimentally (1 σ (β see Figure) averaged 0.07? CFC 0 036 PS 0 009 and at plasma colloid osmotic pressure of 20 5 mmHg CL was estimated to 0 029 during isogravimetry

It is concluded that during isogravimetry at ordinary capillary pressures the transmicrovascular albumin passage is to about 70 per cent due to filtration, whereas only some 30 per cent of transport takes place by diffusion, both events presumably via large pores There was no evidence that transendothelial vesicular transport should to any significant extent contribute to the passage of albumin from vessel to tissue

C 6

Dependence of Arterial Pressure Level and of Metabolic and β Adrenergic Stimuli on Vascular Tone and Dynamic Myogenic Reactivity in the Muscle Microcirculation

By P O GRANDE and P BORGSTRÖM *Department of Physiology University of Lund Sweden*

Recent studies have demonstrated the existence of a rate sensitive (dynamic) component in myogenic microvascular control (Johansson and Mellander 1975 Grände *et al* 1977 1978) which is much more effective than the previously analysed steadystate (static) myogenic response In the present study microvascular tone and dynamic reactivity in response to a standardized increase (40 mmHg) of vascular transmural pressure (P_T) applied at a given rate ($dP_T/dt = 2.5$ mmHg/s) were analysed in skeletal muscle during variation of arterial pressure (AP) and under the influence of metabolic and β-adrenergic stimuli Graded decreases of AP graded metabolic stimulation (muscle exercise) or graded β-adrenergic stimulation (i.a. infusion of adrenaline after α-blockade) all caused graded decreases of microvasular tone until virtual abolition at an AP level of 40 mmHg, exercuse at 0 4 Hz or adrenaline infusion at a rate of 2μg min kg respectively The dynamic constrictor responses to the standardized myogenic stimulus declined in parallel with the decreasing microvascular tone during metabolic and βadrenergic stimulation but was almost fully maintained during decreased AP Microvascular tone at lowered AP could however not be re-established by restoration of static vascular distending pressure to normal which was experimentally produced by decreasing extravascular pressure *Microvascular tone thus is intimately depending on the prevailing* AP but apparently not on its static distension effect but could rather be explained by affects of repetitive dynamic stimulation somehow induced by the AP level Metabolic and β-adrenergic stimuli can effectively adjust microvascular tone during normal AP most likely via their influence on dynamic myogenic reactivity and thereby can contribute to the control of capillary exchange functions Surgical trauma and moderate haemorrhage could abolish microvascular myogenic reactivity but it was almost momentarily restored by β-adrenoceptor blockade Stressinduced adrenaline release might thus explain the absence of myogenic reactivity and that their tone was hardly at all affected by lowered AP or by the mentioned relatively mild metabolic and β-adrenergic stimuli

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C 7

Distribution of LRH Immunoreactivity in Neural and Gastrointestinal Tissues

By H. SELÄNNE, M. TUOVINEN and J. LEPPÄLUOTO, *Department of Physiology, University of Oulu, Finland*

Hypothalamic release and release inhibiting factors or hormones consist of a group of small polypeptides which regulate the release of anterior pituitary hormones. It has been postulated that all peptide hormone-producing cells are derivatives of the neuroectoderm and, hence, distributed in adults in the central nervous system, gastrointestinal tract and skin (Pearse 1976). Therefore, we have measured the concentrations of luteinizing hormone-releasing hormone (LRH) in various tissues of central nervous system and of gastrointestinal tract.

Outbred Sprague-Dawley rats weighing 200–300 g were used. After decapitation specimens of following tissue samples were removed: hypothalamus, cerebrum, cerebellum, spinal cord, pancreas, stomach, duodenum, colon and liver. The tissue specimens were weighed and homogenized in 90% methanol. After centrifugation the supernatants were evaporated and the residues were washed with ml of 0.01 M acetic acid and chloroform. The upper phase was submitted to gel filtration in LH 20 (Pharmacia, Sweden, 1×40 cm column) in 100% methanol and 3 ml fractions were collected, evaporated and LRH was measured by radioimmunoassay (Seppälä, Ranta and Leppäluoto 1974). The fractions containing LRH were submitted to CM-chromatography and assayed as before.

Quantitatively highest LRH immunoreactive concentration was found in hypothalamus 250 pg per mg of wet tissue, while cerebrum had 0.6, spinal cord 0.1 and cerebellum less than 0.01 pg/mg LRH. The immunoreactive LRH concentrations of gastrointestinal tissues varied from less than 0.001 to 1 pg/mg, and was highest in the pancreas, 1.0 pg/mg. In the stomach there was 0.05, duodenum 0.04 and colon 0.01 pg/mg LRH. The LRH immunoreactivity of the liver was non-detectable.

During the course of the purification the LRH immunoreactivity of the hypothalamus, cerebrum, cerebellum, spinal cord, pancreas, stomach, duodenum and colon behaved like synthetic LRH. The main function of LRH is evidently the stimulation of gonadotropin release from the pituitary, which would explain its high concentration in hypothalamus. The role of LRH in gastrointestinal tissues, if any, remains to be established.

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C 8

Cardiorespiratory Effects of Centrally Administered TRH

By F KOIVUSALO I PAAKKARI J LEPPÄLUOTO and H KARPPANEN *Departments of Physiology and Pharmacology University of Oulu Finland*

Thyrotropin releasing hormone (TRH) is found throughout the nervous system (Leppäluoto et al 1978) Centrally administered TRH elevates blood pressure in the cat and rabbit (Delbarre *et al* 1977 and Beale *et al* 1977)

In this study increasing amounts of TRH (3 16 80 400 and 2000 ng per rat) or alternatively saline were injected into the lateral ventricle of urethane-anaesthetized rats Mean blood pressure and heart rate were measured directly from the left femoral artery by means of a pressure transucer a pressure amplifier and a rate computer (Hewlett Packard) Ventilation was measured with a hot wire air flowmeter and the signals integrated with an integrator (Hewlett Packard) to obtain the tidal volume

A dose of 3 ng TRH resulted in a significant increase in blood pressure and heart rate (Fig 1 panel A and B) whereas 16 ng was needed before ventilation rate was increased (data not shown) The increased ventilation rate parallelled a simultaneous decrease in tidal volume and hence there was no significant change in ventilatory minute volume The cornea reflex became positive in all anaesthetized rats after 3 ng TRH but remained negative in the control rats

Our results confirm the previously reported pressor effect of centrally administered TRH and demonstrate that TRH is one of the most potent centrally effective hypertensive agents The lack of change in ventilatory minute volume indicates that respiratory feed-back mechanism work under influence of TRH The physiological phenomena affected by

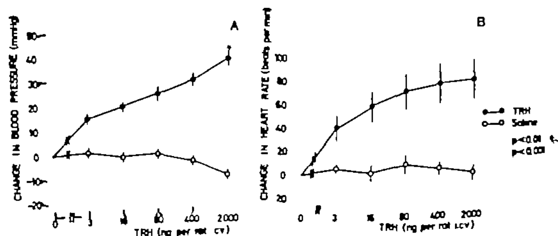


Fig 1 The effect of increasing doses of intracerebroventricularly injected TRH or saline on mean blood pressure (panel A) and heart rate (panel B) Each symbol is given as mean±SE (n=8) and represents the values obtained 10 min after injection. Asterisks indicate a statistical significance between TRH and the corresponding saline injections

TRH injections are controlled by medulla oblongata and spinal cord which is in accordance with the high TRH content of these areas.

This study was supported by grants from the Orion-Yhtyma Foundation

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C 9

Adrenal Medullary Control of Muscular Glycogenolysis in Exercising Rats

By E. A. RICHTER, H GALBO and N J CHRISTENSEN *Institute of Medical Physiology B, University of Copenhagen and Department of Internal Medicine and Endocrinology Herlev Hospital Denmark*

We have previously shown (Galbo *et al* 1978) that in adrenodemedullated rats injected with 6-hydroxydopamine (selectively destroying sympathetic nerve endings) muscular glycogen depletion during exercise is smaller than in control rats

Now we have elucidated if this effect of sympathectomy can be ascribed selectively to the lack of either the adrenal medulla or of the peripheral sympathetic nerve endings

48 male Wistar rats weighing 180–200 g were either adrenodemedullated (DM) or sham operated (C) Two weeks later unilateral exstirpation of the sympathetic chain from above the second lumbar to below the second sacral ganglion was performed During the operation skin temperature on the foot increased .5°C in the denervated and 0.8°C in the intact innervated leg. Seven days after sympathectomy the concentrations of norepinephrine in the sympathectomized soleus and deep vastus lateralis muscles were reduced to 9% and 11 respectively of control values

One week after sympathectomy the rats were either forced to swim with a tail weight (2 of body weight) or were resting controls. 7 DM and 9 C-rats swam 75 min and 8 DM and 8 C-rats swam to exhaustion. Then the rats were stunned by a blow on the head and blood was collected from the carotid artery Samples of the soleus muscles and of the liver were quickly removed.

At rest, concentrations of glucose in blood and of lactate in muscle were similar in DM and C-rats whereas muscular and hepatic glycogen concentrations were higher in DM rats In C-rats blood glucose had increased muscular and hepatic glycogen had decreased but muscular lactate was unchanged after 75 min of swimming. At exhaustion (236 ± 10 min, mean SE) hepatic glycogen had further diminished and blood glucose had decreased below values at rest (P<0 05) In DM-rats blood glucose muscular glycogen and lactate did not change significantly during swimming. Hepatic glycogen was significantly reduced at exhaustion (191 min ± 17 min) but was still higher than in C rats Sympathectomized and control legs did never differ significantly with respect to muscular glycogen and lactate concentrations

C 8

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In this study increasing amounts of TRH (3 16 80 400 and 2 000 ng per rat) or alternatively saline were injected into the lateral ventricle of urethane-anaesthetized rats. Mean blood pressure and heart rate were measured directly from the left femoral artery by means of a pressure transucer a pressure amplifier and a rate computer (Hewlett Packard). Ventilation was measured with a hot wire air flowmeter and the signals integrated with an integrator (Hewlett Packard) to obtain the tidal volume

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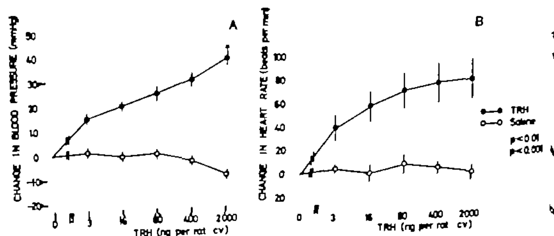


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sistance (FBF being related to muscle mass) with the result that MAP is only moderately affected. Under these conditions stroke volume usually is only affected to a minor extent (1). An increase in HR would, therefore, imply a proportional increase in CO. On this basis it might be concluded that CO is regulated according to muscle mass (and V) involved in exercise.

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C 11

Effects of Renal Nerve Stimulation on Arteriolar Resistances, Ultrafiltration Dynamics and Fluid Reabsorption

By Hermansson, K., M. Larson, Ö. Källskog and M. Wolgast. *Department of Physiology and Medical Biophysics, University of Uppsala, Sweden*

The influence of a Hz and 5 Hz nerve stimulation on the hydrostatic pressure of the renal vasculature and proximal tubule, as well as the nefron filtration (SNGFR) and the nefron effective plasma flow (SNEPF) was investigated in 10 Sprague Dawley rats. During 2 Hz stimulation the plasma flow decreased from 164±7.5 (mean ± SEM) to 138±8.9 nl/min and SNGFR from 54.5± .8 to 46.3± 7 nl/min. This was due to a 30 % increase in both afferent and efferent arteriolar resistance, whereas the glomerular capillary pressure remained fairly constant at about 60 mmHg. The filtration fraction increased somewhat. The mean net driving force for filtration was 15.9 and 13.2 mmHg during these conditions, whereas the hydraulic conductivity remained unchanged at 3.4–3.5 nl/min · mmHg.

The same pattern of response was seen with 5 Hz stimulation except for more marked decreases to 50 % of the controls for SNEPF and SNGFR. The afferent arteriolar resistance increased 2.3 times, whereas the efferent only increased 1.5 times the controls. The fractional proximal reabsorption increased (TF/p from 6.0±0.39 to 7.1±0.48 for control condition and Hz stimulation respectively). The absolute reabsorption decreased however with the decreased SNGFR. It is concluded that sympathetic nerve stimulation may act in direction of fluid conservation by a decreased plasma flow and glomerular filtration and an enhanced fractional fluid reabsorption.

C 12

Arteriolar Resistance and Single Glomerular Blood Flow (SGBF) During a Maximal Tubulo-glomerular Feedback Stimulus in the Amphibian Kidney

By B. E. Persson and A. E. G. Persson. *Department of Physiology and Medical Biophysics, University of Uppsala, Sweden*

It is concluded that in exercising rats 1) adrenal medullary hormones are essential for muscular glycogen breakdown and endurance capacity 2) Sympathetic nerve activity does not significantly enhance muscular glycogenolysis 3) Exhaustion does not necessarily coincide with emptying of muscular glycogen stores

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C 10

Role of Muscle Mass in the Pressor Responses to Graded Muscle Ischemia in Man

By Y SUZUKI and F BONDE PETERSEN *August Krogh Institute University of Copenhagen Denmark*

Graded muscle ischemia produced by arterial occlusion cuffs applied to the thighs during or immediately after bicycle exercise markedly influences cardiovascular function Occlusion 30 sec before end of exercise had the most pronounced effect increasing mean arterial pressure (MAP) cardiac output (CO) and HR significantly above control values during recovery (1 2) This was attributed to a reflex mechanism elicited through the chemoreceptors in the occluded muscles CO is diverted to skin vascular bed probably by baroreceptor activity

In the present experiment forearm blood flow (FBF) V_0 MAP HR and mechanocardiogram (MCG) (based on simultaneous tracing of ECG phonocardiogram and carotid epicutaneous pulse wave) was recorded in 3 subjects during one- or two-legged exercise in the reclined position on a bicycle ergometer Three experimental situations were used 1) rest 7 min bicycle exercise recovery 2) rest 7 min bicycle exercise recovery with occlusion applied during the first 3 min of recovery and 3) essentially as 2) but occlusion applied 30 sec before end of exercise These protocols were repeated twice during two-legged exercise and twice during one legged exercise

Our data confirmed previous findings (1 2) for two-legged exercise In addition MCG revealed that diastolic pressure time index/systolic tension time index (DPTI/TTI) decreased during both experiment 2 and 3 indicating an increased delivery/demand ratio for oxygen to the coronary circulation This is attributed to the increase in MAP observed Relative speed of contraction of the ventricles expressed as preejection period/ejection time (PEP/ET) did not vary

When one- and two-legged exercise was compared similar MAP and MCG were generally observed whether or not occlusion was applied HR was increased by 62 bpm during two-leg exercise and 34 bpm during one leg exercise and 69 and 42 bpm respectively if occlusion was applied Occlusion however increased FBF to levels approximately twice as high during two-leg exercise as during one-leg exercise. The HR reflex appears to be muscle mass dependent while baroreceptors are evidently able to regulate peripheral

The intratubular P_{CO_2} was significantly higher than the P_{CO_2} of the arterial blood and the P_{O_2} of the efferent arteriolar blood was significantly lower than that of the arterial blood The buffer capacity was higher and the bicarbonate concentration slightly lower for the efferent arteriolar blood than for the arterial blood

It is concluded that a P_{CO_2} difference exists across the tubular wall and that the high intratubular P_{CO_2} favours a chemical equilibrium of the carbonic acid-bicarbonate system in the proximal tubular fluid It is supposed that the slightly lowered bicarbonate concentration in the efferent arteriolar blood is an effect of the glomerular ultrafiltration process

C 14

HCO_3^- transport by bullfrog duodenum

By Gunnar Flemström. *Department of Physiology and Medical Biophysics Uni-versity of Uppsala S eden*

Recent studies have shown that gastric antral and fundic surface epithelial cells actively transport HCO_3^- into the gastric lumen. Potent inhibitors of this secretion also decrease the ability of the gastric mucosa to resist intraluminal acid This made it of interest to examine whether the surface epithelial cells in proximal duodenum have a similar HCO transport.

The duodenal bulb and adjacent part of the duodenum was removed from bullfrogs immediately after killing of the animals by decapitation The muscular coat was removed by blunt dissection and the mucosal tube (total length 7–8 mm) mounted between two glass tubes connected to a reservoir The unbuffered luminal solution (5 ml) was gassed with 100% O_2 Each also gave satisfactory circulation of the solution HCO_3^- transport was measured by continuous titration to pH 7 40 with 5 mM HCl solution under automatic control from a pH-stat equipment (Radiometer Copenhagen) A buffered solution (pH 7.28) gassed with 95% O_2 and 5% CO_2 was used on the nutrient side The transepithelial electric potential diffence was measured via two matched calomel electrodes and recorded with a high input impedance mV meter Microscopic examination showed the absence of Brunner's glands in the present preparations.

All preparations alkalinized the luminal surface The steady state values 60 min after mounting of HCO_3^- transport and electric potential difference was 0.51±0 06 μeq h and 11±0 5 mV (means ± SE, n=12) Elevation of nutrient calcium ion concentration from 1.8 to 7 mM significantly ($p<0.01$) increased both the rate of HCO_3^- transport and the electric potential difference (n=7) Addition of 2.4 dinitrophenol to the nutrient side (10^{-4}M) decreased the electric potential difference and almost abolished HCO_3^- transport (n=5)

The results indicate that surface epithelial cells in proximal duodenum alkalinize the luminal surface The stimulation of HCO_3 transport by calcium ions and inhibition by 2:4 dinitrophenol is similar to that previously observed in gastric mucosa in vitro and in vivo Surface epithelial transport of HCO_3^- may contribute to the resistance of the proximal duodenum to intraluminal acid by increasing the pH in the immediate vicinity of the luminal surface

Earlier experiments have shown the existence of a tubulo-glomerular feedback control in the Amphiuma kidney that can reduce single glomerular filtration rate (SNGFR) at increased delivery of fluid to the distal nephron This decrease in SNGFR could be caused by changes in afferent arterioles resistance (R_a) efferent arterioles resistance (R_e) or SGBF Therefore these parameters were measured at zero and 50 nl/min distal perfusion rate where the high flow releases a maximal reduction of SNGFR To calculate R_a R_e and SGBF it was necessary to measure arterial blood pressure (AP) glomerular capillary pressure (GCP) Bowman space presure (BSP) SNGFR afferent and efferent colloid-osmotic pressures (π and π)

Perfusion nl/min	AP cm H_2O	GCP cm H_2O	BSP cm H_2O	PCP cm H_2O	SNGFR nl/min	π a cm H_2O	π e cm H_2O
0	26.0	18 7	7 0	7 5	18 0	7 7	11 9
50	25 2	14 1	4 8	7 4	7 1	7.8	11 4

At maximal feedback reduction of SNGFR from 18 0 nl/min to 7 1 nl/min SGBF was reduced from 170 nl/min to 40 nl/min R_e did not change significantly (0 157 and 0 235 cm H_2O min/nl) while R_a increased significantly from 0 090 to 0 284 cm H_2O min/nl Thus the SNGFR and SGBF reduction at maximal feedback response is mediated via an increased resistance in the afferent arteriole

C 13

Pco_2 of the Superificial Structures in the Rat Kidney

By M SOHTELL *Department of Physiology and Medical Biophysics Biomedical Center University of Uppsala Sweden*

By the ultrafiltration process in the kidney the proximal tubules are offered a load of bicarbonate The mechanisms underlying the tubular bicarbonate reabsorption have been the subject of much discussion One of these mechanisms generally favoured is the active hydrogen ion transport into the tubular lumen According to this hypothesis the H and HCO_3-ions react and form carbonic acid This acid is then dehydrated forming water and carbon dioxide which are reabsorbed The rate of this reabsorption may be influenced by various factors such as *the rate of delivery of H* the dehydration rate and the carbon dioxide diffusion across the tubular wall

Recordings in vivo of the carbon dioxide tension of the proximal tubular fluid and of the efferent arteriolar blood were performed with Pco_2 microelectrodes in the rat kidney The buffer lines of the efferent arteriolar blood and systemic arterial blood were determined with an ultramicro equilibration system and the acid-base status of the systemic arterial blood was measured

The results support the hypothesis (Folkow et al 1974) that genetic hypertension is associated with a narrower lumen a thicker media and a greater contractility in the precapillary resistance vessels, but with little difference in the structure or contractile abilities of post-capillary resistance vessels

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C 16

Sodium Balance and Dopamine Excretion in Spontaneously Hypertensive Rats (SHR)

By H HERLITZ, S LUNDIN M HENNING, S E. RICKSTEN G GÖTHBERG M HALLBÄCK and G BERGLUND *Departments of Physiology Pharmacology and Med Clin I University of Göteborg Sweden*

In SHR sympathetic overdrive in combination with thickening of the arterioles peripherally precedes the increase in blood pressure (Lundgren 1974) In another strain of such rats the development of hypertension has been found to be associated with sodium retention (Dietz et al 1975) Sodium balance studies in SHR and normotensive control rats (NCR) revealed a lowered urinary sodium excretion in rats 6–7 weeks of age considered to be in the borderline phase of hypertension (Herlitz et al 1978) There was however at this age no true sodium retention since faecal sodium content was doubled

Recently a dopamine system has been shown to be of importance in the regulation of sodium balance since this catecholamine has pronounced natriuretic effect (Faucheux et al 19[illegible])

To evaluate the effect of increased sodium intake on sodium handling in SHR NCR and Wistar Kyoto rats (WKR) a study with a higher salt intake was performed (16 mmol sodium/100 g food). Rats 6–7 weeks of age were kept in metabolic cages and fed standard diet (5 mmol sodium/100 g) for two weeks after which time the high salt diet was introduced and continued for another week. During these time periods sodium intake urinary and faecal sodium excretion and dopamine excretion were determined (see Table I)

Urinary sodium excretion increased markedly in all groups receiving the high salt diet which demonstrates that also the prehypertensive kidney can excrete a sodium load. There was however still a significantly lower value for SHR which could be due to enhanced mineralocorticoid activity or to increased sympathetic tone Faecal sodium content did not increase during the high salt diet indicating that the enhanced faecal sodium excretion in SHR is not secondary to a diminished ability of the kidneys to handle salt The increased dopamine excretion might be a mechanism by which the organism protects its sodium homeostasis.

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C 15

Structure and Function of Arterial and Venous Resistance Vessels In Spontaneously Hypertensive and Normotensive rats

C AALKJÆR and M J MULVANY *Biophysics Institute Aarhus University Denmark*

Segments of arterial resistance vessels (i d −190 μm) and their corresponding veins have been taken from a precisely defined location in the mesenteric bed of normotensive (WKY) and Spontaneously hypertensive (SHR) rats (Mulvany Hansen and Aalkjær 1978) There were mounted in vitro on a myograph capable of directly registering their wall tension while their internal circumference was controlled All vessels were set to a *normalized internal circumference* L_o *close to the value for which the active wall* tension was a maximum (Mulvany & Halpern 1977) At this internal circumference the media thickness m_o, was measured by light microscopy The active wall tension ΔT_o in response to a K rich solution was then determined The average results from 18 pairs of vessels are presented in Fig 1 The active pressure Δp the vessels could have produced in vivo was calculated using Laplace s law ($\Delta p = 2\pi \Delta T_o / L_o$) and was (Mean±S E.) for the arteries SHR 274 81±9 85 mmHg WKY 167 59±7 14 mmHg and for the veins SHR. 20 33±1 58 mmHg WKY 19 10±2 18 mmHg

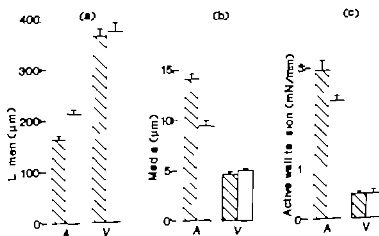


Fig. 1 (a) Normalized lumen diameter $l = L_o/\pi$ (b) media thickness at L_o, m_o (c) active wall tension ΔT of pairs of arterial (A) and corresponding venous (V) resistance vessels taken from 9 SHR rats (hatched columns) and 9 control WKY rats (open columns) Columns heights show means and bars show S E. s *indicates significant difference between SHR and WKY values ($P<0.02$)

The results support the hypothesis (Folkow et al. 1974) that genetic hypertension is associated with a narrower lumen a thicker media and a greater contractility in the precapillary resistance vessels, but with little difference in the structure or contractile abilities of post-capillary resistance vessels.

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C 16

Sodium Balance and Dopamine Excretion in Spontaneously Hypertensi e Rats (SHR)

By H Herlitz, S Lundin M Henning S E Ricksten G Göthberg M Hallbäck and G Berglund *Departments of Physiology Pharmacology and Med Clin. I University of Goteborg Sweden*

In SHR sympathetic overdrive in combination with thickening of the arterioles peripherally ...dels the increase in blood pressure (Lundgren 1974) In another strain of such rats the development of hypertension has been found to be associated with sodium retention (...ds et al. 1975) Sodium balance studies in SHR and normotensi e control rats (NCR) revealed a lowered urinary sodium excretion in rats 6-7 weeks of age considered to be in the borderline phase of hypertension (Herlitz et al 1978) There was however at this age no true sodium retention since faecal sodium content was doubled

Recently a dopamine system has been shown to be of importance in the regulation of sodium balance since this catecholamine has pronounced natriuretic effect (Faucheux et al 197-)

To evaluate the effect of increased sodium intake on sodium handling in SHR NCR and Wistar Kyoto rats (WKR) a study with a higher salt intake was performed (16 mmol sodium/100 g food). Rats 6-7 weeks of age were kept in metabolic cages and fed standard diet (5 mmol sodium/100 g) for two weeks after which time the high salt diet was introduced and continued for another week. During these time periods sodium intake urinary and faecal sodium excretion and dopamine excretion were determined (see Table I)

Urinary sodium excretion increased markedly in all groups recei ing the high salt diet ...ch demonstrates that also the prehypertensive kidney can excrete a sodium load. There ...however still a significantly lower value for SHR which could be due to enhanced ...eralocorticoid activity or to increased sympathetic tone Faecal sodium content did not ...crease during the high salt diet indicating that the enhanced faecal sodium excretion in ...R is not secondary to a diminished ability of the kidneys to handle salt The increased ...al excretion might be a mechanism by which the organism protects its sodium ...ostasis

TABLE I *Fractional sodium* excretion in urine and faeces in NCR, SHR and WKR receiving two different diets Urinary dopamine excretion during standard diet Fractional urinary excretion=urinary excretion/intake ×100

	Standard diet			High salt diet	
	Fractional urinary sodium excretion %	Fractional faecal sodium excretion %	Dopamine excretion (μg/day)	Fractional urinary sodium excretion %	Fractional faecal sodium excretion %
NCR (n=10)	54±2	12±1	1 3±0 1	88±2	8±1
SHR (n=10)	28±2	32±	2 1±0 1	78±2	15±1
WKR (n=5)	59±4	16±3	1 ±0 1	84±2	13±1

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C 17

Erythrocytic Nucleoside Triphosphates Inhibit Oxygen-linked Binding of Carbon Dioxide by Carp Hemoglobin

By ROY E WEBER *Department of Zoophysiology University of Aarhus Denmark*

It has long been known that in mammalian blood CO_2 is transported partly as carbamate formed by direct combination with hemoglobin More recent studies have shown that CO binds at the α amino groups (valine residues) of the α and β chains of hemoglobin resulting in inhibitory O_2 linkage (reduction in O_2 affinity) (*cf* Kilmartin and Rossi-Bernardi 1971) When protonated the α amino groups of the β chains are however also implicated in binding of anionic diphosphoglycerate (DPG) which regulates erythrocytes O affinity so that competition for this site explains the antagonism observed by Bauer (1969) between CO_2 and DPG binding in human hemoglobin No data appear to be available on the specific effect of CO_2 and its interaction with cofactors in fish Although CO tensions normally are low in aquatic animals many fresh water systems are hypercarbic resulting in high internal CO_2 tensions In fishes moreover nucleoside triphosphates (NTP) substitute DPG as modulators of O_2 affinity in carps changes in both guanosine triphosphate (GTP and ATP serve to adapt blood O_2 affinity to water O_2 tension (Weber and Lykkeboe 1978) These considerations prompted this investigation of CO_2 and NTP interactions in carp hemoglobin

At pH values above 7 7 CO_2 distinctly depresses O_2 affinity and increases cooperativity of stripped (cofactor free) carp hemoglobin (see Fig 1) While GTP and ATP decrease O_2 affinity this effect increases with decreasing pH resulting in increased Bohr shifts (Δlog

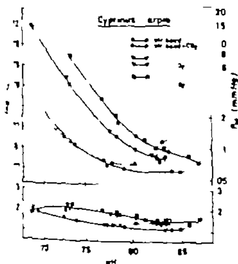


Fig. 1 Influence of CO_2 on half saturation O_2 tension (P_{50}) and cooperativity coefficient (n) of stripped carp hemoglobin in the absence of cofactor (○ ●) and in the presence of ATP (▽ ▼) and GTP (□, ■) Open symbols and solid curves, no CO_2 solid symbols and broken curves, 1% CO_2 (~7.3 mmHg) Temperature, 20°C solvent Tris buffer ionic strength 0.06 hemoglobin concentration, 0.09 mM (tetramer basis): molar ratio of ATP and GTP over hemoglobin, ~4

Δ log P_{50}/ΔpH) These data imply that as in mammals, CO_2 and cofactors bind at uncharged $-NH_2$ and protonated $-NH$ groups respectively Significantly however ATP as well as GTP completely obliterate the CO_2 effect, except at high pH (above 8.2) (Fig 1) This suggests that carbamate will normally play an insignificant role in CO_2 transport in carp blood, and that CO_2 will have little direct influence on O_2 transport The complete suppression of O_2-linked CO_2 binding by cofactors contrasts to mammalian hemoglobin where DPG reduces the CO_2 effect It implies that the β chains must be the main sites for carbamate formation in carps This tallies neatly with the finding (H Ise and Braunitzer 1968) that in the chains of carp hemoglobin, the N-terminal valine residue is substituted by N-acetylated serine

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C 18

Glucose Transport by the Integument of a Polychaete Worm

By J Gomme, August Krogh Institute Zoophysiological Laboratory A University of Copenhagen Denmark

The epidermis of the brackishwater polychaete *Nereis diversicolor* accumulates exogenous D-glucose from natural environmental concentrations, i e <10 μM (Ahearn & Gomme

TABLE I Fractional sodium excretion in urine and faeces in NCR, SHR and WKR receiving two different diets Urinary dopamine excretion during standard diet Fractional urinary excretion=urinary excretion/intake ×100

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partial denervation. We have repeated this experiment with electrophysiological techniques on the mammalian peripheral sympathetic system.

The right superior cervical ganglion of the guinea-pig was partially denervated by cutting the preganglionic fibres that run dorsal to *arteria subclavia* (about 75%). The synaptic contribution from the remaining intact axons (running ventral to the artery in *ansa subclavia*) was measured by recording intracellularly the synaptic responses in neurones in the superior cervical ganglion while stimulating the *ansa subclavia in vitro*. In normal ganglia, the median number of fibres from this source innervating each neurone was 2.5, whereas in ganglia examined seven days after partial denervation the median increased to 3.0 (n=4.04, Wilcoxon test). The median amplitude of the compound excitatory postsynaptic potential (e.p.s.p.) also increased from 6.8 mV in normal ganglia to 13.0 mV after partial denervation. Since an increase in the number of fibres innervating each neurone cannot be explained by supersensitivity or by more transmitter being released from each ending, much of the increase in the synaptic response is probably caused by sprouting of preganglionic axons with formation of new synaptic connexions.

In further experiments, a stimulating electrode was placed on the cervical sympathetic trunk for 1 hr immediately after the partial denervation. To test whether the electrode caused any nerve injury, the current was not turned on in a series of control animals. The results of these experiments were not significantly different from those obtained after partial denervation alone (α=0.15). In the remaining animals, electrical stimulation was carried out for 1 hr at supramaximal intensity while observing the sympathetic end organ responses (3 s trains of biphasic 0.5 ms square waves, ±10 V, ±0.5 mA, 20 Hz, train rate 1/6 Hz). Electrical stimulation caused a substantial net increase in the synaptic responses evoked from *ansa subclavia* after seven days. The median number of fibres innervating each cell increased from 2.7 in ganglia that had been partially denervated and sham stimulated to 3.8 in ganglia examined after partial denervation and electrical stimulation (n=4.002). The median e.p.s.p. amplitude increased from 11.0 mV to 18.1 mV.

These results show that in the superior cervical ganglion, sprouting after partial denervation can be enhanced by a period of preganglionic nerve stimulation.

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C 20

Developmental Changes in the Pattern of Innervation of Focally Innervated Rat Skeletal Muscle

B. S. POCKETT, P. M. SANDSET and J. K. S. JANSEN. *Institute of Physiology and Institute of Anatomy, University of Oslo, Norway*

In mammals the pattern of innervation of skeletal muscle is dramatically reorganized during early post natal development (Redfern 1970). The significance and universality of this process is not known.

1975 Gomme in prep) The aim of the present study was to characterize D-glucos transport into this tissue

By incubating groups of animals for 1–4 min in artificial sea water with 1–1000 µ radiolabelled D-glucose influx across the apical membrane of the epidermal cells could b determined The identity of the rate-limiting barrier was verified by suitable contr experiments The cuticle a 1–2 µm protein polysaccharide covering on the epidermis w found to restrict diffusion of substrate to the membrane in a way comparable to but mor pronounced than of an unstirred layer of medium Diffusion of D-glucose through the inte cellular epidermal spaces was negligible as compared to uptake into the cells

The data fit the assumption of a one-component stereo-specific saturable D-glucos influx ($K \sim 100\,\mu M$ $J_{max} \sim 10$ mol cm h) across the apical membrane 100 µM phlorizi completely blocks D-glucose uptake while phloretin is a less efficient inhibitor D-glucos influx is Na -dependent as shown by substitution of Na with choline

Net uptake of D-glucose from low concentrations (~1 µM) determined from the rate removal of D-glucose from the incubation medium was found to be of the same magnitu as influx Determination of net uptake was technically impossible at higher concentration

Under natural conditions D-glucose is rapidly phosphorylated and metabolized up uptake into the epidermis Sampling of blood and coelomic fluid during incubation wi labelled substrate demonstrated trans-epithelial movement of D-glucose to be negligib and of the order of magnitude of trans-epithelial uptake of D-mannitol from a simil concentration

It is suggested that D-glucose transport across the apical membrane of *Nereis* epiderm bears resemblance to that across the apical membrane of epithelia with significant tran epithelial D-glucose movement (mammalian intestine and kidney tubule Cestoda epidermis) and thus is distinct from D-glucose uptake into non polar cells In *Nerei* epidermis however trans-epithelial D-glucose uptake is negligible The transport syste in the apical membrane like that in the membrane of non-polar cells seems to serve onl the nutrition of the transporting tissue

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C 19

Sprouting after Partial Denervation in the Superior Cervical Ganglion: Effect of Preganglionic Nerve Stimulation

By JAN MÆHLEN and ARILD NJA *Institute of Physiology University of Oslo Norway*

Cutting some of the nerve fibres innervating a target causes sprouting of the remaining intact axons Hoffman (1952) showed histologically that in rat skeletal muscle the number of subsequent sprouts could be increased by electrical nerve stimulation at the time of

with sound, light, vibration, smell and cold, the EC-conditions being the same as reported by Diamond et al.

The cerebral cortices of formalin-perfused brains were investigated after Klüver Barrera staining. The relative magnitude R = cortical depth/whole brain weight was measured in the somesthetic and occipital areas. This magnitude can be suggested to reveal the capacity of information handling better than absolute values of cortical depth (see Jerison 1970). The results showed that 1) no significant difference in R was to be found between the IC and ICS-groups, 2) a significant decrease in R occurred in the ICS-group as compared with EC-group in part of the lateral occipital area 3) a significant increase in R was found in the EC-group as compared with the IC-group in both lateral and medial occipital areas. The absolute values of cortical depth yielded no significant differences.

It is considered that in some of the cortical areas an optimal sensory stimulus pressure can be measured which results in an enhancement of the cortical growth. In these areas excess stimulation as well as impoverishment results in an inhibition of the cortical growth.

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C 22

Intracellular release of Ca^{2+} May Counteract the Negative Inotropic effect of Acidosis in Myocardium

By H. Gesser and O. Poupa, *Department of Zoophysiology, University of Aarhus, Denmark, and Department of Clinical Physiology, University of Göteborg, Sweden*

H⁺-ions allegedly inhibit the myocardial force development by competing with Ca^{2+}-ions for sites on the contractile proteins (Katz and Hecht 1968). The heart of some species (e.g. slender turtle snake) in contrast to other (cod, trout, carp) overcome this inhibition by some as yet unexplained compensatory mechanism (Poupa and Johansen 1975, Gesser and Poupa 1978).

A flux of Ca^{2+} from the mitochondria to the cytoplasm triggered by acidosis has been postulated as a compensatory mechanism (Gesser and Poupa 1978).

The present study on flounder myocardial strips demonstrates a compensation elicited by increased PCO_2, but not by a decrease in HCO_3^-. Analysis of ^{45}Ca efflux rates from flounder heart strips show nearly the double efflux rates when the myocardium is exposed to 15% CO_2 compared to 3% CO_2. These results are strongly suggesting that recovery of myocardial contractility during acidosis depend on an increase in the cytosolic Ca^{2+} activity.

We have examined the fl dig brevis I muscle of prehatched chickens This is a focall innervated muscle Staining for Ach-esterase demonstrated a band of end-plates along th center of the muscle fibres From the time of the histological appearance of end-plat esterase (13 days) there is only a single esterase site in each muscle fibre

In 16 and 17 days old embryos more than one axon made synaptic contact with eac end plate When the nerve was stimulated at threshold intracellular recording showed unitary epp On increasing the stimulus intensity the amplitude of the epp increasec indicating recruitment of one or more additional axons innervating the same fibre Th individual components of the compound epps always had the same time course In additio we saw smaller and slower synaptic potentials presumably due to electrical couplin between muscle fibres in the young chickens Compound epps were also seen afte stimulation of the muscle nerve at its exit from the spinal column The threshold c activation and the conduction times of the functional motor axons varied by less than 5 percent in the 16 day old embryos This corresponds to a distinct group of large axons h the muscle nerve

Just before hatching at day ?0 the muscle fibres were innervated only by single axons, a they are in older chickens

We conclude that focally innervated chick muscle goes through an early developmenta stage where the muscle fibres are probably polyneuronally innervated The elimination o the redundant innervation is completed during the last week before hatching.

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C 21

Reduction of the Depth of the Cerebral Cortex in the Mouse due to Excess Sensory Stimulation

By R M BERGSTROM O F C LINDROOS and M L A RIITTINEN *Institute o Physiology University of Helsinki Finland*

Brain stimulation in an enriched environment as compared with impoverished cage condi tions increases the depth of the cerebral cortex (see e g Diamond et al 1972) Since it can be calculated (Bergström and Nevanlinna 1972) that excess activation of neural networks reduces their information handling capacity it can be suggested that overstimulation in the young mouse would reduce the *amount of cortical substance* which is responsible for the informational capacity Therefore experiments were made with three groups of mice (totally 62 F_1-hybrids of inbred strains C3H/BR and C57BL/6J age 7 days) living for 39 days in three different environments impoverished (IC) enriched (EC) and overstimulated (ICS) The specific environmental condition for the ICS-group was applied for 2 hours daily and for EC-group for ?–?4 hours daily ICS-conditions included excess stimulation

...al, light, vibration smell and cold, the EC-conditions being the same as reported by Diamond et al.

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C 22

Intracellular release of Ca^{2+} May Counteract the Negative Inotropic effect of Acidosis in Flounders

By H. Gesser and O. Poupa, Department of Zoophysiology, University of Aarhus, Denmark, and Department of Clinical Physiology, University of Göteborg, Sweden

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C 23

Cardiovascular Control Mechanisms During Isometric Contractions— – a One-leg Strength Training Model

By ELSE NYGAARD STEVEN LEWIS JORGE SANCHEZ and BENGT SALTIN *August Krogh Institute University of Copenhagen Denmark*

The classical concept has been that the blood pressure (BP) and heart rate (HR) responses to isometric contraction are dependent on the relative force (percentage of maximal voluntary contraction force MVC) exerted by the contracting muscles (Lind and McNicol 1967) However there is recent evidence that the responses elicited may also in some cases be related to the absolute force exerted (Mitchell et al 1977)

The present study was designed to determine whether absolute or relative force exerted is of primary importance and to examine the interplay of central versus peripheral control mechanisms for the BP and HR responses This was accomplished by testing the knee extensor muscles of each leg of 9 healthy men before and after the muscles of one leg had been strength trained The subjects performed 120 maximal isometric knee extension contractions/day lasting 5 sec each 5 days/week for 9 weeks Direct BP (brachial artery) HR and rectified electromyography (EMG) of both legs were recorded continuously during 2 min isometric knee extension contractions with each leg

MVC before and after training was 56±11 and 68±9 kg ($p<0.05$) in the trained leg (TL) and 56±6 and 59±8 kg in the untrained leg (UTL) respectively No differences in BP HR and EMG were observed during contraction with TL or UTL when each was tested at the same *relative load* (40% MVC) before and after training After training mean BP HR and EMG were lower than before training with TL and UTL when each was tested at the same *absolute load* And when TL was compared with UTL after training at the same absolute load all above variables were lower with TL (see Table which gives mean ± SD at min 2 test + = $p<0.05$)

		TL		UTL		TL vs. UTL		
Mean BP	pre	152 (23)	+	152 (16)	+	TL	133 (5)	
(mmHg)	post	131 (9)		133 (7)		UTL	143 (10)	
HR	pre	108 (16)	+	117 (19)	+	TL	97 (12)	+
(beats/min)	post	97 (13)		106 (18)		UTL	108 (18)	
EMG	pre	1 6 (0 2)		1.8 (0 5)		TL	1 4 (0.3)	+
(arb units)	post	1.3 (1 2)		1 6 (0 4)		UTL	1 9 (0.3)	

The present results clearly demonstrate that BP and HR responses to isometric contraction with a given muscle group are primarily dependent on the relative rather than the absolute force exerted by the contracting muscles The pronounced reductions in BP HR

[illegible] EMG during contraction with both TL and UTL at the same absolute load after training suggests that strength training reduces the influence from the motor cortex (central command). However the lower BP HR and EMG responses of TL as compared with UTL at the same absolute load also point to peripheral factors influencing cardiovascular regulation.

One possible interpretation of the present findings is that although central command may be lower with both TL and UTL after training there is an additional reduction in central command with TL. The hypertrophy of trained muscle and the increased maximal force development in our subjects (muscle biopsy and computerized tomography scanning data) may require fewer motor units to be activated at the same absolute load in TL.

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C 24

Discrimination of Enantiomers in Olfaction

By H. MUSTAPARTA, M. E. ANGST and G. N. LANIER. *Institute of Biology Odense University Denmark*

Discrimination of enantiomers in olfaction is of interest because of their identical chemical properties, but steric differences. The present study deals with receptor discrimination of pheromone enantiomers which cause dramatical differences in the behavior of bark beetles and thus have played a decisive role in the evolution of the reception. One species *Ips paraconfusus* Lanier produce the (+)-ipsdienol(2-metyl-6-methylene 2 7-octan-4-ol) which is important for the attraction while the (−)form has no effect (Vité pers. comm.). Another species *I. pini* shows variations of two geographical isolated populations. The western (USA) population living together with *I. paraconfusus* produce the (−)form, while the eastern one produce mixture of the two isomers. The attractive effect of the two enantiomers seems to be in accordance with the production (Lanier in progress). The present study was made to disclose the underlying receptor discrimination which account for these behavioral effects.

We have recorded responses from single olfactory cells in these species while stimulating with both enantiomers and the racemic mixture. All cells activated by one form, were also activated by the other. By using a syringe olfactometer and double series of each stimulant significant differences of the stimulation effect appeared. One enantiomer was commonly ten times more effective than the other. Thus, in *I. pini* most of the ipsdienol cells were more sensitive to the (−)form than to the (+)form, while the opposite appeared to be the case for *I. paraconfusus*. The racemic mixture showed an intermediate effect. These

differences in receptor sensitivities may explain the behavioral differences of *I paraconfusus* and the western *I pini* The behaviour of the eastern *I pini* is more complicated to explain It seems that the other "ipdienol cells" play a role in the central pathways resulting in their preferences for the (+)form or the racemic mixture In general the discrimination of these enantiomers seems to be due to both an interaction on the receptors as well as an interaction in the central pathways

C 25

Role of C3–C4 Propriospinal Neurones in Forelimb Movements in the Cat

By B ALSTERMARK A LUNDBERG U NORRSELL and E SYBIRSKA *Department of Physiology University of Göteborg Sweden*

Higher centres excite forelimb motoneurones disynaptically via propriospinal neurones (PN) which take origin in the C3–C4 segments These PNs are characterized by monosynaptic convergence mainly from descending pathways e g corticospinal rubrospinal tectospinal and bulbospinal tracts but also from forelimb afferents (Illert et al 1977 1978) Disynaptic inhibition evoked from the same sources may serve as feed forward inhibition giving spatial selectivity in activation of the PNs (Illert et al 1975) The C3–C4 PNs presumably form a premotoneuronal spinal centre which integrates information from many sources We have attempted to elucidate their role by investigating movements in cats after spinal cord lesions

Motor proficiency was tested by means of the ability to take food from the bottom of a cylinder (height 40 mm diameter 30 mm) placed vertically on the floor or horizontally 150 mm above the floor level Using these tests Gorska and Sybirska (in press) found severe impairment of contralateral forelimb movements after transection of the pyramid and destruction of the red nucleus

The dorsal or ventral part of the lateral funiculus was transected in C2 or C5 The dorsal C2 lesion was made in order to interrupt rubrospinal and corticospinal connexions both to the PNs and the forelimb segments The dorsal C5 lesion interrupts the connexions to the forelimb segments without interfering with the input to the C3–C4 PNs or their ventrally located axonal projections to forelimb motoneurones (Illert et al 1978) The dorsal C2 lesion gave severe motor defects similar to those found after lesions of the pyramid and the red nucleus (Gorska and Sybirska in press) In contrast animals with dorsal C5 lesions placed the forepaw rapidly and accurately into the cylinder already 10 days after surgery movements of digits were deficient during the entire postsurgical periods (3 months)

After a ventral C5 transection the cats lifted the forelimb in the direction of the cylinder but there was marked ataxia placing the forepaw in the cylinder often required several adjusting movements Once having reached the cylinder the animals performed the movements of the digits and the forepaw supination required to take the food. No defects of forelimb movements were observed after a ventral C2 lesion which interrupted other fibre systems with the same transverse location as the propriospinal axons

The results suggest that the C3–C4 propriospinal system is utilized in precise rapid movements of the forelimb. Fine motor control of digits seems to operate through interneuronal systems located within the forelimb segments.

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C 26

Effect of Amiloride on the Mechanosensitivity of Hair Cells in the Lateral Line System of Xenopus laevis

By FINN JØRGENSEN, *Institute of Physiology, Odense University, Denmark*

In the model for the transducer mechanism of hair cells Davis (1965) suggested that a leakage current across the transducer membrane was modulated by changes in membrane conductance and that these were governed by movements of the sensory hair of the cells.

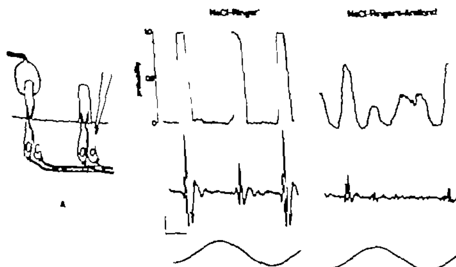


Fig. 1. A. A schematic drawing of a stitch showing two of the neuromasts each represented by two hair cells of opposite polarity. Action potentials were recorded with glass microelectrodes close to the [illegible] neuromast and neuromasts more caudally were mechanically stimulated by a moving glassball attached to a loudspeaker. B. The bottom traces show the driving voltage to the loudspeaker (20 Hz). This voltage also triggered the sweep of the averaging computer. The upper traces show the probability of occurrence of afferent spikes relative to the phase of the mechanical stimulation obtained in Ringer solution and in Ringer + 9 · 10^{-4} M Amiloride. The duration of the square pulse triggered by the spikes and averaged [illegible] times, was 5 msec. The middle traces show the averaged spikes obtained in the two solutions. The amplitude of the spikes did not change during the experiment. Calibration: [illegible] V and 10 msec.

Ca and K ions have been suggested as charge carriers across the transducer membrane (Sand 1975 Russel and Sellick 1976) A leakage current across the transducer membrane has not been demonstrated but might depend upon an energy dependent transport of NaCl across the sensory epithelium

The effect of Amiloride on the mechanosensitivity of the free standing hair cells in the lateral line system of Necturus mac has been investigated Fig 1B shows an example of this effect on the probability for occurrence of afferent spikes from the stitch relative to the phase of optimal mechanical stimulation (POS) The change in height of POS reflects the effect of Amiloride In 31 experiments the average change in POS was 45% ± 4% (SEM). Substitution of Na by choline or removal of NaCl did not significantly affect the mechanosensitivity In solutions of low or zero NaCl 9×10^{-4} M Amiloride reduced the POS by 38% ± 5% (SEM) n=29 The effect of Amiloride was reversible and at concentrations of 1 2×10 M the afferent spikes were not phase locked to the mechanical stimulation

The effect of Amiloride on the mechanosensitivity might suggest that the permeability of the outward facing membrane of the hair cell to Na is of importance to the ionic mechanism of the hair cells The lack of effect by the removal of Na from the solution however questions the specificity of Amiloride in this system in contrast to findings in frog skin

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C 27

Transfer Properties of the Slowly Adapting Stretch Receptor of the Crayfish Abdomen

By GUNNAR SYDNES *Institute of physiology University of Oslo Norway*

I have studied the transfer properties of the slowly adapting stretch receptor of the crayfish abdomen (see Alexandrowicz 1967) by introducing small sinusoidal variations to the length of the receptor muscle There was reason to believe that the isolation procedure used by previous investigators (Borsellino et al 1965 Brown & Stein 1966) might interfere with the properties of the receptor (see Njå & Walloe 1973) Therefore the receptor was left in situ Reflex input to the receptor was eliminated by cutting its central connection and the cut end of the nerve was drawn into a suction electrode for registration of receptor activity

The sinusoidal variation on the input was generated by a feed-backcontrolled electromagnetic stretcher The movements of that stretcher was transfered to a glass rod which was fastened to the 4 tergite of the isolated abdomen thus stretching the receptor of the third abdominal segment In most experiments the *sinusoidal variation* was superimposed on a steady flexion in the joint of about 30° This regularly resulted in a

mean firing-rate of 10–15 imp/sec. The preparation was bathed in Ringer solution with temperature about 11°C

The position in the joint investigated was measured by means of two position transducers Signals from these together with the nerve signals were fed into a NORD-1 computer Using a procedure finding the 0 and 1 harmonic analytically both the mean and the 1 harmonic of both input and output could be rapidly calculated

I first investigated the linearity of the receptor by applying signals with different amplitudes to the input of the receptor The amplification of the system proved to be surprisingly constant over a large range of input amplitudes Accordingly the system should be well suited for transfer property investigations

The transfer properties of the receptor organ were investigated by stimulating the receptor with low-amplitude signals with different modulation frequencies. Small amplitudes were used to prevent phase locking of the receptor response It was then possible to measure the amplitude of the impulse train even when the modulation frequency was well above the mean firing-rate of the stretch receptor

The amplification of the system as function of modulation frequency showed a regular behaviour from one experiment to another There was a characteristic maximum in amplification at about 5 Hz modulation frequency the maximum being about 3 times the amplification found for very low modulation frequencies When the modulation frequency was increased above 5 Hz, the amplification always fell rapidly towards zero

Within the limits of linearity the receptor of our preparation shows a characteristic behaviour also found by previous investigators on isolated receptors (Borsellino et al. 1965) However our results suggest that more stable and reproducible results can be obtained by leaving the receptor in situ than has previously been found in isolated preparations

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C 28

Effect of Membrane Polarization in Motoneurones on Timing of Antidromic Invasion

By J LIPSKI Department of Physiology University of Goteborg Sweden

Changes in latency of antidromic action potentials recorded extracellularly from single neurones have been used as an indirect measure of neuronal excitability (Merrill 1974 Lipski et al 1977 1978) This approach was based on the observation that the delay between the initial segment (IS) and soma-dendritic (SD) spikes recorded intracellularly from motoneurones is shortened by depolarization and lengthened by hyperpolarization of the cell body (Brock et al 1953)

The latency to the extracellularly recorded antidromic action potentials might be used to estimate the level of membrane polarization in a more quantitative way provided that the relation between the IS–SD transition time and some polarization were known in greater detail It should also be important to know whether the IS–SD delay were solely responsible for the variation in the latency of the extracellularly recorded antidromic spikes

To analyse the timing of antidromic invasion delays between stimuli applied to the ventral root and the onsets of the IS and SD spikes were measured while recording intracellularly from lumbar motoneurones Depolarizing and hyperpolarizing current pulses (15–30 ms 1–20 nA) were applied to displace the membrane potential the membrane potential and the input resistance were calculated using the spike height method (Frank and Fuortes 1956) In all motoneurones studied there was approximately linear relationship between the IS–SD delay and soma polarization for both depolarizing and hyperpolarizing directions However latency increase during hyperpolarization was larger (0 40 ms/10 mV±0 18 n=12) than the rate of latency decrease during depolarization (0 16 ms/10 mV±0 12 n=14) The larger changes of the IS–SD delay in hyperpolarizing direction could be explained in part by the conductance increase occurring with hyperpolarization (anomalous rectification) The delay from the stimulus to the IS spike changed in the same direction as the IS–SD delay but the rate of changes was smaller (mean 0 1? ms/10 mV for hyperpolarizing and 0 05 ms/10 mV for depolarizing currents) This suggests that the antidromic invasion of the IS region and/or conduction velocity in the proximal part of the axon also may be influenced by membrane potential in motoneurone soma

The shifts in latency of SD spikes in motoneurones evoked by polarizing current pulses are comparable to the fluctuations of latency of antidromic invasion observed in brainstem neurones whose membrane potential was modified by synaptic inputs (cf Merrill 1974 Lipski et al 1977 1978) The present results suggest that recording of antidromic latency changes may be a particularly sensitive method for detecting inhibition of neurones in extracellular studies

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C 29

Differentiated Adrenergic Responses of Human Oviductal Muscle Layers to Noradrenaline and Transmural Nerve Stimulation

By B Lindblom B Ljung and L. Hamberger *Departments of Physiology and Obstetrics and Gynecology University of Göteborg Sweden*

Studies on ovum transport in the human oviduct have demonstrated a fast passage through the distal segments followed by a delay for 2–3 days at the proximal end of the ampullary

portion (Croxatto et al 1977). A sphincter-like function may therefore be attributed to the ampullary-isthmic junction (AIJ) which has a rich supply of adrenergic nerves (Owman et al 1967). *In vitro* studies on small strips from the two major smooth muscle layers of the human AIJ (Lindblom et al 1978) revealed that adrenaline as well as noradrenaline (NA) cause inhibition of the spontaneous activity in strips from the inner circular layer but increase contractile activity in strips from the external, longitudinal layer The present investigation was initiated to compare the effects of NA with those of transmural nerve stimulation (TNS) on this *in vitro* system

Human oviducts were obtained at operation and millimeter wide muscle strips were prepared from both the circular and longitudinal layers The preparations were mounted in an organ bath and contractile activity was recorded isometrically Atropin was added to the medium to avoid possible influence of cholinergic nerves

TNS (6 Hz, 15 V 4 ms) caused relaxation of strips from the circular layer but increased activity of longitudinal strips. Responses of similar character and magnitude were elicited by addition of NA (1 0 μ M) β-adrenoceptor blockade by propranolol abolished the inhibitory response of the circular muscle to NA and augmented the stimulatory effects of NA and TNS in the longitudinal strips The inhibitory response of the circular strips to TNS was not constantly blocked by propranolol, which seems to indicate a non-adrenergic inhibitory neurotransmitter The α-adrenoceptor antagonist phenoxybenzamine blocked the stimulatory response of the longitud nal specimens to both TNS and NA. Pretreatment with tetrodotoxin abolished the effects of TNS but not the response to NA.

The present results give additional evidence for a differentiated distribution of α- and β-adrenoceptors in the smooth muscle layers of the human oviductal isthmus The longitudinal layer exhibits marked dominance of α-receptors whereas in the circular layer the β receptors are predominant. If this concept is applicable in the *in vivo* situation a properly timed adrenergic activation might play a facilitating role for the passage of the ovum through the AIJ by an increased contractility of the longitudinal muscle layer in combination with a relaxation of the internal circular layer Furthermore such relaxation may be obtained by a non-adrenergic neurogenic mechanism

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C 30

Adrenergic and Cholinergic Responses to Transmural Nerve Stimulation in Rat Uterus in Vitro

By S E. DAHLEN and P HEDQVIST *From the Department of Physiology Karolinska Institutet Stockholm Sweden*

The latency to the extracellularly recorded antidromic action potentials might be used to estimate the level of membrane polarization in a more quantitative way provided that the relation between the IS–SD transition time and some polarization were known in greater detail It should also be important to know whether the IS–SD delay were solely responsible for the variation in the latency of the extracellularly recorded antidromic spikes.

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(Abrahamsson, Glise and Glise 1978) nociceptive stimuli in the gastroduodenal area was found to suppress gastric motility by activation of vagal non-adrenergic inhibitory fibres to the stomach. The present study was designed to explore the possible reflex pathway for vagal gastric inhibition in connection with nociceptive stimuli in the small intestine

In anesthetized cats gastric motility was recorded by a volume method. Defined nociceptive jejunal stimuli (Diathermic lesions or mechanical stimulation) promptly induced a pronounced gastric relaxation which persisted after administration of atropine and guanethidine as well as after adrenalectomy. The response was abolished by vagotomy or by spinal cord transection at the cervical level and was temporary blocked by spinal anesthesia.

Afferent electric stimulation of cut mesenteric or splanchnic nerves also elicited gastric reflex relaxation with characteristics similar to that obtained on nociceptive stimulation.

It is concluded that nociceptive intestinal stimuli induce suppression of gastric motility via a reflex utilizing sympathetic afferents and ascending spinal pathways as the afferent link. The vagal non-adrenergic inhibitory system constitutes the efferent part of the reflex. The described intestino-gastric spino-vagal reflex is suggested to be important for the pronounced gastric inhibition in paralytic ileus.

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C 32

Characteristics of the Sympathetic Nervous Activity in Awake Normotensive and Hypertensive Rats

By S.-E. RICKSTEN and P. THORÉN. *Department of Physiology, University of Göteborg, Sweden*

The sympathetic nervous discharge was recorded from splanchnic branches in awake adult normotensive Wistar Kyoto WK (NCR) rats or spontaneously hypertensive rats SHR of the Okamoto strain

During Nembutal® anaesthesia, small bipolar silver electrodes were placed on the sympathetic branch and isolated with Wacker silicone rubber (Sil Gel 604) according to the technique described by (Schad and Seller 1975). The animals were allowed to recover for at least 4 hours. Then the nervous activity, heart rate and blood pressure were recorded when the animals were fully awake. The normotensive rats showed at rest a very stabile level of blood pressure, heart rate and nervous activity. In comparison SHR showed a much more varying level of sympathetic nervous activity. In both SHR and WK there were a close correlation between changes in sympathetic nervous activity and heart rate

The reflex response in heart rate, blood pressure and nervous activity was examined

There is considerable morphological evidence that the rat myometrium receives both cholinergic and adrenergic innervation However the function of these nerves is still largely unsettled In the present communication we have studied some effects of transmural nerve stimulation in isolated uterine horns of rats

Strips or segments of uterine horns under different hormonal conditions were placed in organ baths containing Mg^{2} free Ringer solution gassed with 6 5% CO_2 in O_2 kept at 37°C Spontaneous contractions and responses to transmural nerve stimulation (1–10 Hz, pulse duration 0 2–1 0 ms supramaximal voltage) were recorded isotonically (load 0.5 g)

Transmural stimulation of uterine horns from nonpregnant rats produced inhibition of spontaneous activity whether the animals had received estrogen treatment for two weeks or not This inhibitory response was annulled by tetrodotoxin (0 3 μM) and guanethidine (1 μM) but left unaffected by practolol (2–4 μM) phenoxybenzamine (1–4 μM) or phentolamine (1–5 μM) This suggests that the inhibitory response was due to stimulation of adrenergic nerves acting on β_2-adrenoceptors

Atropine (0 3–1 0 μM) did not significantly affect the spontaneous activity but often enhanced the inhibitory response possibly due to removal of a cholinergic excitatory response In order to unmask a cholinergic excitatory response the spontaneous activity had to be eliminated This was achieved by either of the following treatments 5–10 mM $MgCl_2$ and 5 mM $CaCl_2$ or 1 mM $MgCl_2$ at decreased bath temperature (32°C) or inhibition of prostaglandin synthesis After discontinuation of spontaneous contractions transmural nerve stimulation of uterine horns from nonpregnant rats produced contractions that were blocked by tetrodotoxin (0.3 μM) or atropine (0.3–1 0 μM) but left unchanged by adrenergic blocking agents

In contrast transmural nerve stimulation of longitudinal strips from rats late in pregnancy (day 20–21) elicited spontaneouslike contractions whether the preparations were left spontaneously active or not However these responses were unaffected by tetrodotoxin (0 16–3 1 μM) or drugs which block adrenergic as well as cholinergic receptors The abscence of nerve induced uterine responses in the 20–21 day pregnant rat, may at least in part be explained by the reduction of innervation density that has been reported late in pregnancy

Our study indicates that the non pregnant rat uterus has both cholinergic excitatory and adrenergic inhibitory responses which however seem to disappear late in pregnancy

C 31

Vagal Inhibition of Gastric Motility Elicited from the Small Intestine

By H ABRAHAMSSON and H GLISE. Department of Physiology University of Göteborg and Department of Medicine II Sahlgren s Hospital Sweden

Gastrointestinal motility is greatly impaired by intraabdominal nociceptive stimulation e g abdominal surgery An increased adrenergic nervous activity is commonly regarded as the main pathogenetic factor in such paralytic ileus However in a recent study

(Abrahamsson, Glise and Glise 1978) nociceptive stimuli in the gastroduodenal area was found to suppress gastric motility by activation of vagal non-adrenergic inhibitory fibres to the stomach. The present study was designed to explore the possible reflex pathway for vagal gastric inhibition in connection with nociceptive stimuli in the small intestine.

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The reflex response in heart rate, blood pressure and nervous activity was examined

during blood volume expension Upon 10% increase in blood volume the splancnic nervous activity decreased 42% in SHR and 33% in NCR The same transfusion increased blood pressure 5 1 mmHg in SHR and 9 ? mmHg in NCR Thus upon volume load the hypertensive animals seem to *increase the blood pressure less* in comparison with WK because the sympatethic nervous activity is *decreased more* in SHR than in NCR. The only reasonable explanation for this finding is a hyperreactive volume receptor mechanism in the spontaneously hypertensive rats

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C 33

Diurnal Variation of Immunoreactive LRH Concentrations in Hypothalamus Preoptic Area and Amygdala in Male Rats

By M TUOMINEN H SELANNE and J LEPPALUOTO *Department of Physiology University of Oulu Finland*

Serum LH levels show random fluctuation but no clear circadian rhythm (Kalra and Kalra 1977) This would indicate that hypothalamic LRH concentrations do not undergo distinct circadian rhythm Therefore we have measured hypothalamic LRH concentrations at various time of day Because of conflicting reports about the effects of electrical and chemical stimuli to amygdala on gonadotropin release we also measured the LRH concentrations of amygdala

Male rats were housed for 3 weeks in a silent room with lights on from 6 AM to 6 PM Five or six rats were rapidly decapitated at 4 hrs intervals The tissue samples were excised extracted with methanol evaporated dissolved in buffer and measured in a specific radioimmunoassay for LRH (Seppälä, Ranta and Leppäluoto 1974) Hypothalamic samples included the median eminence and its surrounding between mamillary bodies and optic chiasma and they weighed from 8 to 16 mg Preoptic areas weighed from 5 to 11 mg and amygdalas from 17 to 25 mg.

Hypothalamic and preoptic LRH concentrations did not show any statistical variations either in the light or dark period of the day Hypothalamic LRH concentrations varied from 646 to 970 pg per mg of wet tissue and preoptic ones from 70 to 160 pg/mg The highest LRH concentrations in amygdala was found at 4 PM 11.5 ± 1.8 pg/mg and the lowest at 4 AM 2.3 ± 1.5 pg/mg ($p > 0\ 01$ to 4 PM values)

The absence of circadian rhythm either in hypothalamic or preoptic LRH concentrations in male rats agrees whith the findings of Kalra and Kalra (1976) Further our finding of

diurnal fluctuation of LRH concentration in amygdala might be in some relation with that of FSH in serum (Kalra and Kalra 1977). This result does not conflict with the finding that stimulation of amygdala leads to gonadotropin release (Kawakami & Terasawa 1974).

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C 34

The Effect of Norepinephrine on Hippocampal Pyramidal Cells *in vitro*

By I. A. LANGMOEN and M. SEGAL. *Institute of Neurophysiology, University of Oslo, Norway*

Extracellular recordings from hippocampal pyramidal cells have suggested that norepinephrine (NE) is a putative inhibitory transmitter in the hippocampus (Segal and Bloom, 1974). The mechanism for this inhibition, however, is still unkown. The aim of the present study was to investigate by intracellular recordings the action of NE on the hippocampal pyramidal cells.

Recordings were obtained from CA1 pyramidal cells using hippocampal slices maintained *in vitro* (Skrede and Westgaard, 1971). Prior to the application of NE, control responses to depolarizing and hyperpolarizing current pulses passed through the recording electrode were obtained for 1–8 minutes. Droplets (ca. 1 nanoliter) of NE (10^{-4}–10^{-6} M) were then applied from a pipette positioned close to the recording electrode.

After application of NE the cell hyperpolarized up to 4–5 mV accompanied by an up to 25–30% reduction in resistance. The two effects were always closely associated with a reduction in the number of spikes elicited by depolarizing current pulses. In spontaneously firing cells, NE either reduced the firing rate or completely abolished spontaneous activity. The resting potential, membrane resistance and spontaneous and evoked activity all recovered within a period of 10 minutes after application. Similar results were obtained in preliminary experiments in which NE was administered iontophoretically.

In order to exclude the possibility that the inhibitory effect of NE was mediated through interneurons, applications of NE were made after reducing calcium and increasing magnesium levels in the incubation solution. While this procedure completely blocked synaptic transmission, NE still had similar effects on the pyramidal cells.

Our results support the concept that NE serves as an inhibitory transmitter on the CA1 hippocampal pyramidal cells. The mechanism for this inhibition seems to be a combination of increased conductance and hyperpolarization of the cell membrane.

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C 35

Synaptic Connections to Perigeniculate Neurones

By GUNILLA AHLSÉN and SIVERT LINDSTRÖM *Department of Physiology University of Göteborg Göteborg Sweden*

Postsynaptic inhibition can be evoked in principal cells of the lateral geniculate body by electrical stimulation of the primary visual cortex It has been suggested that this type of inhibition is mediated by a subcortical recurrent inhibitory pathway involving axon collaterals of principal cells and an inhibitory interneurone (Andersen Eccles and Sears 1964 Burke and Sefton 1966) In keeping with this idea it has recently been found that principal cells issue axon collaterals dorsally to the geniculate body in the perigeniculate region (Ahlsén Lindström and Sybirska 1978) Neurones in this region have now been studied to determine if they might serve as interneurones in the suggested recurrent pathway to principal cells

The experiments were performed on adult cats under pentobarbital anaesthesia Stimulation electrodes were placed in the optic tract and in the primary visual cortex Extra and intracellular recordings have been obtained from 38 perigeniculate neurones identified by their location 100–400 μm above the lateral geniculate body The cells were synaptically activated by stimulation of the optic tract and the visual cortex Usually a train of spikes could be evoked by both types of stimulation The latency of the first spike indicates a monosynaptic linkage from the cortex and a disynaptic linkage from the optic tract as would be expected if the cells were excited via axon collaterals of principal cells. Collision tests performed with a few intracellularly recorded cells have confirmed that the first part of the excitation is mediated by such collaterals

The later spikes in the train seems to be due to a different excitatory component which presumably is mediated by slowly conducting corticofugal fibres In all tested cells the excitation was followed by a longlasting inhibition both when the cortex and the optic tract were stimulated The properties of this inhibition indicates that it is mediated by mutual inhibitory connections between perigeniculate neurones All these synaptic effects on perigeniculate neurones closely match the behavior of the inhibitory potentials evoked in principal cells by similar stimulations Thus the present observations give strong support to the hypothesis that perigeniculate neurones are interposed as interneurones in a recurrent inhibitory pathway to principal cells

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C 36

Motor Activity Retards Kindling Rate in Rats

By SVEIN GRAHNSTEDT *Institute of Physiology University of Bergen Norway*

Several reports have suggested that increased CNS activation is inhibitory to the occurrence of epileptic seizures. It has also been shown that reduced stress and thus possibly reduced activation, will facilitate the development of epileptic susceptibility In this experiment, activation expressed as motor activity vs inactivity was investigated regarding influence on development of epileptic susceptibility

Repeated electrical stimulation of the brain with low current intensities leads to a progressive decrease in seizure threshold Goddard named this process kindling in his original report. The kindling paradigm provides an excellent model of epilepsy that is easy to control, and interference with normal behavior is minimal

For this experiment rats were implanted with bipolar electrodes in the right amygdala All animals were handled every day from one week before surgery till testing started two weeks after surgery The animals were kept on a 12 hour light-dark cycle in a sound-shielded room to minimize external interference with the experiment Each day all animals were allowed at least three minutes in the testing chamber for habituation and scoring of spontaneous activity before stimulation. After having shown the desired activity for at least three seconds, the rats were stimulated.

Seizure development was scored according to Racine Number of days of stimulation before showing a class 5 seizure that is a full motor seizure with loss of equilibrium was measured. The rats stimulated when showing motor activity were significantly slower than those stimulated when quiet, in reaching criteria

This experiment has shown that normal behavior can influence the development of epileptic susceptibility in rats. In particular motor activity retarded the kindling rate The results are in agreement with earlier findings that has shown that increased activation is inhibitory to development of epileptic susceptibility as well as occurrence of seizures in susceptible individuals.

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C 35

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By GUNILLA AHLSÉN and SIVERT LINDSTRÖM *Department of Physiology University of Göteborg Göteborg Sweden*

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It is concluded that a decrease in gall-bladder transepithelial net Na-transport of about 80% induced by a small serosal hydrostatic pressure of 4.5 cm water results mainly from a decrease in J^{Na}_{ms}, and not from an increase in paracellular leakage Thus the present results support the hypothesis that gall-bladder transepithelial Na-transport is determined by mechanical forces.

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D 2

Effects of "Physiological" Concentrations of Adrenaline on Perfused Fish Gills

By I WAHLQVIST and S NILSSON Department of Zoophysiology U iversity of Göteborg S eden

In teleost fish, catecholamines are stored in and released from chromaffin tissue in the anterior part of the kidney (head kidney) Stress produced by keeping the animal in air for 15 min, induces a release of amines and the plasma concentration of adrenaline increases from 0 04 to 0.23 μM in blood sampled from the caudal vessels (Nilsson *et al* 1976). New preliminary results suggest that the plasma concentration in blood sampled from a gill artery *i.e* closer to the source of adrenaline release might be even higher

These changes in concentration of adrenaline in plasma may affect the contractile force of the cod heart. EC_{50} = 0.23 μM for the positive inotropic effect of adrenaline on the cod



Fig 1 Increase in the rate of flow through isolated perfused cod gill arches produced by increasing concentrations of adrenaline. The shaded area indicates the "physiological" range of adrenaline concentration in plasma from blood sampled in the caudal vessels of "non-stressed" and "stressed" fish. Number of estimations in each point = 10–14, variation shown as standard error (S.E).

DEMONSTRATIONS

D 1

Sensitivity of Gall-bladder Na-transport to a small Serosal Hydrostatic Pressure

By O FREDERIKSEN *Institute for Experimental Medicine University of Copenhagen Copenhagen Denmark*

Two fundamentally different hypotheses have been proposed to explain transepithelial transport of Na salts in isosmotically transporting epithelia such as the gall-bladder The one postulates Na-transport by an ion- or salt pump localized in the lateral cell membrane a resulting local hypertonicity in the lateral intercellular space is held to be responsible for consequent passive osmotic transport of water (Diamond & Bossert 1967) According to the alternative hypothesis transcellular isosmotic transport proceeds by transport of Na-salts and water as *fluid* volumes in separate subcellular compartments by means of mechanical (contractile) forces (Frederiksen & Leyssac 1969) The former theory predicts that active Na-transport *should be rather insensitive to small serosal hydrostatic pressures,* while the latter may allow a very high sensitivity of the active Na transport to such pressures Thus a distinction between the two concepts might be possible from estimates of the pressure sensitivity of the mucosal-to-serosal Na-flux (J^{Na}_{ms}) and the passive serosal-to-mucosal Na-backflux (J^{Na}_{sm}) provided that gross leakage of the epithelium due to serosal pressure elevation can be avoided

In the present investigation rabbit gall bladders were mounted as flat sheets in *Ussing* chambers *A thin soft nylonnet supported the mucosal surface Transepithelial potential* difference (P d) Resistance (R_T) and J^{Na}_{ms} or J^{Na}_{sm} (as ^{22}Na flux) was determined A serosal pressure of 4 5 cm water was applied for 40 min by elevating the serosal half-chamber Unidirectional Na-fluxes are in terms of neq min cm ^{2} All data are given as means ±SEM

The serosal pressure caused J^{Na}_{ms} to decrease from 453± 6 to 233±15 (n=6) the halftime was 6 min In the recovery period J^{Na}_{ms} returned to 456±27 the halftime was 4.5 min J^{Na}_{sm} (n=6) increased transiently within *5 min of pressure application from 137±7 to 308±38* and then decreased rapidly again to near control values after 40 min J^{Na}_{sm} was 177±9 and in the recovery period 132±9 The serosal pressure caused R to increase from 63 7±3 8 to 80 3±2 9 ohm cm² (n=12) After 40 min recovery R was 66.8±4 1 ohm cm² P d. showed only small changes typically a reversible decrease of about 0 5 mV from control values of 3 mV (mucosa positive) was observed during pressure application

rats of group A and C were further unilaterally sympathectomized by surgical extirpation of the sympathetic chain from above the 2nd lumbar to below the 2nd sacral ganglion. The rats of group D were left intact (sham-operated) Sympathectomy (with or without adrenodemedullation) results in a 90 % reduction in the noradrenaline content of hind limb muscles (Richter Galbo and Christensen 1978) Half of the rats in each group (T-rats) were subjected to a 12 week physical training programme consisting of swimming in water maintained at 33-34°C, up to 5.5 hours/day 4 days/week The remaining rats served as sedentary controls (C-rats) 72 hours after the last training session, the rats were weighed and the gastrocnemius muscles were excised bilaterally under pentobarbital anesthesia and immediately frozen in liquid nitrogen for subsequent analysis of succinate dehydrogenase (SDH, EC 1.3 99 1) activity as an indicator of oxidative potential

Irrespective of the operative treatment the weight of the gastrocnemius muscle was lower in T-as opposed to C-rats (−15 % $p<0.001$) However gastrocnemius weight relative to body eight were equal for all rats. The SDH activities for the differently treated hind limbs ere as follows (µmol×g⁻ wet weight)×min) (means ± SE) *Shamoperated* 11.9±0.4 (T ₋₁₁) vs. 8 1±0.3 (C₋₁₀) +46 % $p<0.001$ *Intact adrenal medulla sympathectomized* 9 6±0.6 (T ₋) vs. 7 2±0.5 (C₋₁₁) +33 % $p<0.01$ *adrenodemedullated intact sympathetic chain* 10 6±0 4 (T ₋) vs 7 8±0.3 (C₋₁₀) +35 % $p<0.001$ and *adrenodemedullated sympathectomized* 10 4±0 6 (T ₋) vs. 7.₋±0 6 (C₋ +45 % $p<0.01$

Although a tendency towards a somewhat greater increase in SDH activity in the shamoperated rats can be observed it is concluded that an intact sympatho-adrenal system is not necessary for a marked oxidative enzymatic adaptation of skeletal muscle to increased activity to take place

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D 4

Correlation Between Tension and Structure in a Smooth Muscle During Phasic Contractions

By Keld Hornbech Svendsen *Institute of Biophysics University of Aarhus Aarhus Denmark*

The structure of smooth muscles is still relativly unknown and in published works the muscles have only been investigated under steady state conditions (Vibert et al. 1972) The purpose of the work reported here has been to give information about structural changes in a smooth muscle during phasic contractions where the tension show both contracting and relaxed phases.

atrium (Holmgren 1977) There are also some indirect indications that circulating catecholamines may contribute to the overall control of blood pressure in the cod (Wahlqvist and Nilsson 1977)

The vascular resistance in teleost gills is in contrast to the systemic resistance reduced by catecholamines (for references see e g Wood 1974) The present study was undertaken to examine the possible control of the branchial vasculature by physiological" plasma concentrations of adrenaline

Isolated gill arches from Atlantic cod *Gadus morhua* were perfused at a constant pressure with a cod Ringer s solution containing increasing concentrations of adrenaline. Adrenaline produced a bi phasic change in flow rate a small initial decrease was followed by an increase in perfusion rate The initial decrease can be abolished by phentolamine 1 μM

Concentrations of adrenaline similar to those measured in plasma during stress" may have pronounced effects on the vascular resistance of cod gills (Fig. 1) The range of adrenaline concentrations in "non stressed and "stressed' fish could be even higher than indicated by the figure with higher levels of adrenaline close to the head kidney The possibility of a considerably lower plasma concentration of adrenaline in non-stressed" fish under normal non laboratory conditions must also be remembered

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D 3

Significance of the Sympatho-adrenal System for the Exercise-induced Enzymatic Adaptation of Skeletal Muscle

By J HENRIKSSON J SVEDENHAG E A RICHTER and H GALBO *Department of Physiology III Karolinska Institutet Stockholm Sweden and the Department of Medical Physiology b University of Copenhagen Denmark*

The stimuli triggering the adaptive changes in skeletal muscle oxidative potential to different levels of physical activity are unknown Based on studies with daily injections of isoprenaline as well as with prolonged beta-blockade in rats (Harri and Valtola 1975 Harri 1977) the idea has been put forward that specific activation of beta-receptors on the muscle fibres trigger the enzymatic adaptation The purpose of the present study was to further evaluate this possibility

88 male Sprague-Dawley rats (130 g) were randomly divided into four groups (A–D) In group A and B the rats were adrenodemedullated bilaterally by electrocoagulation The

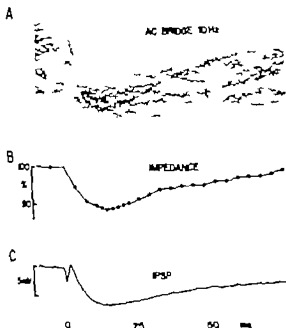


Fig. 1 *A*. 35 superimposed sweeps of the voltage deflections resulting from 0.15 nA 10 Hz Ac current through the recording electrode during the IPSP *B* Relative impedance of the cell, as measured from part A Plotted in percent of resting impedance *C* The timecourse of the IPSP

The conductance change underlying the IPSP was studied by sending a 10 Hz AC current through the intracellular recording electrode By superimposing the records from 20 to 50 such sweeps a picture as the one shown in Fig. 1 A was obtained. From such pictures the impedance was measured in per cent of resting impedance.

There seemed not to be any big difference in the time course of the IPSP and in the impedance change In the cell presented in Fig 1 both the conductance increase and the IPSP were largest after 15 ms. Similar results were obtained from other cells with the peak in conductance however usually 3–4 ms before the maximal amplitude of the IPSP When the cells were hyperpolarized to the equilibrium potential of the IPSP orthodromically evoked EPSP's were only shunted during the period of largest conductance increase (up to 15-25 ms) The probability of firing on orthodromic stimulation could however be reduced for a longer period (up to 60 ms)

The hyperpolarization and the conductance change in recurrent inhibition on hippocampal pyramidal cells show similar time course Our data suggest that the hyperpolarization and the conductance increase cooperatively inhibit the cell during the period of largest conductance change In the later part of the IPSP however inhibition seems to be produced mainly by the hyperpolarization

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After dissection the ABRM muscle from Mytilus Edulis was placed in seawater containing 5-HT For activation a 7 s electrical stimulation was used (Cornelius and Lowy 1978)

The x ray diffraction method gives a diffraction pattern with two prominent reflections namely the meridional 144 A reflection from the periodic structure of myosin filaments and the intensity distribution along the equatorial direction from the planer arrangement of actin filaments (e g 2-dimensional electrondensity projected in a plane at right angle to the longitudinal axis of the muscle) The innermost part of the equatorial distribution provides *information about variations* in the electrondensity at the outer part of the actin filaments, e g decoration with HMM–SI but is relatively insensitive to changes in the actin filament packing.

The observations show that while development of maximum tension and the following relaxation each takes about 10 s the change of structure takes place during the very first second and after the stimulation is finished it takes 30–40 s before the structure returns to a relaxed state The experiments also showed that the diffracted intensity was insensitive to *transitions between* phasic contracting and tonic states *If no 5-HT* was added to the seawater the resting state could give diffracted intensities similar to those obtained from contracted states My interpretation is that a constant fraction of HMM–SI molecules can be attached to actin filaments even if their contribution to the developed tension are varying

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D 5

On the Time-course of Recurrent Inhibition in Hippocampal Pyramidal Cells in vitro

By I A LANGMOEN and R DINGLEDINE *Institute of Neurophysiology University of Oslo Norway*

Recurrent inhibition on the hippocampal pyramidal cells is produced by activation of basket cells which have their synapses on the somata of pyramidal cells (Andersen Eccles and Løyning 1964) Synaptic inhibition is supposed to work through a hyperpolarization of the cell membrane as well as a conductance increase of the cell membrane which inhibit loading of its capacitative elements

In hippocampal pyramidal cells there are no data available comparing the hyperpolarization with the underlying conductance change The aim of the present study was to study the time course of the two events and investigate which of the factors plays the most important role in the inhibition of the spike discharges

Intracellular recordings were obtained from the hippocampal pyramidal cells *in vitro* (Skrede and Westgaard 1971) IPSPs were evoked by subthreshold antidromic stimulation

distance away from the tooth and its supporting tissues (Linden 1978). Recent studies in cats have demonstrated that the discharge from the intraoral mechanoreceptors can be reduced by electrical stimulation of sympathetic fibres to the area (Anderson and Linden 1977). This effect could result from either movement of the tooth secondary to vascular changes (Aars 1978) or to more direct influences on the receptors. As a first step to studying these mechanisms, we have investigated the effect of electrical stimulation of the cat cervical sympathetic nerve on longitudinal and transverse mo ements of the canine tooth.

The cats were anaesthetized with sodium pentobarbitone and tracheotomized. Blood pressure was recorded in a femoral artery and body temperature was maintained using a thermostatically controlled blanket. Longitudinal and labial/palatal movements of the left upper canine were determined by ultrasonic transit time technique (Lerand 1970) with one set of crystals fixed to the tooth the other to the maxilla or to a frame supporting the head.

In five of six cats the canine intruded a maximum of 5–15 μm during sympathetic stimulation (5 V 1 ms 1 20 Hz 30 s periods); extrusion was seen in one. Three canines moved in a labial direction, while the others responded with a palatal movement. These longitudinal and transverse movements were usually evoked by stimulation of high-threshold fibres and could be blocked by phentolamine (2–4 mg/kg). In only one cat, excitation of low-threshold (1.5 V 0.02 ms) fibres in the sympathetic nerve induced extrusion and a palatal movement which was blocked by atropine 0.5 mg/kg, whereas activity in the higher threshold fibres evoked intrusion and a post-stimulatory palatal movement. In another cat a 70 Hz 1 ms stimulation caused a labial movement at 2.5 V and a palatal movement at 5 V.

Our results demonstrate that the cat canine will move in response to sympathetic nerve stimulation and suggest that the movements are mediated mostly by adrenergic more rarely by cholinergic vascular receptors. The direction and extent of movement differs from one cat to another and can be complex.

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D 8

Mitochondria in Rat Intestinal Epithelium

By V. L. KINNULA and I. HASSINEN *Departments of Physiology and Medical Biochemistry University of Oulu Finland*

Because of their high turnover rate (48 h in the rat) the intestinal epithelial cells provide an interesting system for the regulation research of the energy metabolism.

D 6

Spatial Analysis of the Freeze Trapped Brain Provides for Temporal Resolution of an Event. Metabolic Electrical and Blood Flow Changes During Spreading Depression

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Spreading depression (SD) has been studied in terms of changes of energy metabolism blood flow and extracellular potassium concentration (K_e) The SD wave was elicited in nembutal anesthetized craniotomized rats by means of mechanical stimulation in the left frontal cortex and the progression of the wave was followed by a number of suitably spaced cortical electrodes recording either K_e or DC potential The brain was freeze trapped during the progression of the SD wave by funnel freezing with liquid nitrogen Prior to freezing a bolus of ^{14}C butanol was injected in a femoral vein and blood was sampled continuously during a 10 sec period until moment of freezing The frozen brain was sectioned in coronal slices of a thickness of 1 2 mm Right and left side cortex in these slices were dissected at −20°C and extracted with Lowry-technique for measurements of PCr ATP ADP AMP lactate and pyruvate ^{14}C activity was measured for calculation of regional blood flow

Assuming no regional differences in the rat brain cortex with respect to response to the SD wave the spatial analysis of the freeze trapped brain gives a temporal resolution of the SD wave The results may be summarized as follows The change of K_e during the SD wave last approximately 1 min with a sharp rise demarcating the wave front followed by a plateau and then a gradual decline to normal K Related to this time scale changes observed in regional metabolite concentrations and blood flow have a wider extension. Expressed as % of the contralateral region of the unaffected cortex there is a flow decrease of 10–15% immediately preceding the SD wave of an extension of about 1 min coinciding with a decrease in $\frac{ATP}{ADP}$ ratio (10%) as well as a 100% increase in the lactate/pyruvate ratio The sharp rise in potassium is accompanied by a massive increase in blood flow (100–150%) now coinciding with a normalization of the ATP/ADP ratio and an abrupt 5-fold decrease in lactate/pyruvate ratio The flow changes as well as the redox changes normalize somewhat slower than the extracellular potassium concentration

D 7

Movements of the Cat Canine Tooth in Response to Sympathetic Nerve Stimulation

By R. W A LINDEN and H AARS *Department of Physiology Guy's Hospital Medical School London England and Department of Physiology and Biochemistry Dental Faculty University of Oslo Norway*

Activity of intraoral mechanoreceptors that respond to forces applied to teeth are relayed to the brain stem through trigeminal afferent fibres These receptors include both periodontal mechanoreceptors and Type P" mechanoreceptors whose site is thought to be some

The effect of intracranial stimulation of the seventh cranial nerve (n facialis–n intermedius) on the blood flow of the eye and the tongue was studied in rabbits The nerve was electrically stimulated with a stereotaxic technique: pulse intensity 6–14 V duration 1 msec, and frequency 40–50 Hz. Regional blood flow in the eye and the tongue was measured with radioactively labelled microspheres.

Stimulation caused a 212±39% (n=18) (p<0 001) increase in choroidal blood flow on the stimulated side as compared to the control side Flow rates in the ciliary processes and the iris increased by 53±19% (n=18) (p<0 02) and 1 1±_5% (n=18) (p<0 001) respectively Retinal blood flow also tended to increase during stimulation. The blood flow of the mucous membrane of the anterior part of the tongue increased by 210±64% (n=17) (p<0 01) on the stimulated side The blood flow of the corresponding muscle layer of the tongue also increased but with a great variability in the results. The duration of the vasodilation of the eye after a period of stimulation was 30–60 min while in the tongue it was about 10 min Neither the increase in blood flow of the eye nor that of the tongue could adequately be abolished by cholinergic blockade with biperoden (5–10 mg/kg b w) or by ganglionic blockade with hexamethonium (50–100 mg/kg b w) Transsection of the trigeminal nerve prior to stimulation of the facial nerve did not prevent the vasodilation

The phenomenon of atropine-resistant vasodilation in the tongue as a result of corda tympani stimulation is well-known and recent studies by Hellekant (1977) indicate that it is due mainly to activation of nerves originating in the CNS and not in the geniculate ganglion, that is to nerves which seem to be efferent. It is also known that close arterial injection of acetylcholine causes marked but short lasting vasodilation in the choroid in rabbits (Bill 1962)

The efferent autonomic nerves known so far in the intermedio-facial nerve are cholinergic and have synapses in the pterygopalatine or submandibular ganglia. Such efferent nerves cannot explain the present results. The results rather indicate that the intracranial part of the intermedio-facial nerve contains unconventional efferent or afferent fibres that on stimulation release a vasodilating substance in the eye and the tongue that is not acetylcholine

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D 10

Turnover of Noradrenaline Dopamine and 5-Hydroxytryptamine in the CNS under the Influence of 4-Aminopyridine

By NILS-ERIK ANDÉN and STEFAN LEANDER. *Departments of Pharmacology Universities of Goteborg and Lund S eden*

4-Aminopyridine (4-AP) enhances nerve impulse evoked release of both acetylcholine and noradrenaline from peripheral nerves (Leander et al. 1977 Lundh et al 1977) 4-AP also

TABLE I Protein yield cytochrome concentrations succinate dehydrogenase activity and oxygen consumption using succinate as a substrate in mitochondria of intestinal epithelial cells. Values are mean values ± S E. from 12–14 determinations

Protein yield mg/100 g body wt	7 ±0.57
Cytochrome aa_3 nmol/mg protein	0.29±0.02
Cytochrome b nmol/mg protein	0 28±0.02
Cytochromes c+c nmol/mg protein	0.38±0.03
Cytochrome aa_3, Cytochromes c+c	1 1.3
Succinate dehydrogenase μmol/min/mg protein	
Actual	0 22±0 02
Maximal	0.55±0.03
Oxygen consumption natoms/min/mg protein	
State 4	18.9±5.3
State 3	25.3±2.1
Respiratory control ratio State 3/State 4	1 4±0 06

In the present preliminary work conditions of the isolation of rat intestinal epithelial mitochondria and their general properties were determined Intestinal epithelial cells of Sprague-Dawley rats were separated principally according to Harrison and Webster (1969) but using 0 3 M mannitol—10 mM Tris—5 mM EDTA—3 mM MgCl solution pH 7 4 The cells were sedimented and intestinal mitochondria were isolated using the same medium *Mitochondrial cytochromes were determined in 1 M sucrose at liquid nitrogen temperature* (Wilson 1967) Absorption coefficients of De Jonge and Hülsman (1973) were used for calculating the cytochrome concentrations Both actual and maximum activity of succinate dehydrogenase were determined by the method of King (1967) by using substrate activation (Kimura *et al* 1967) Mitochondrial oxygen consumption was determined using a Clark type oxygen electrode The results are represented in Table I

The present results also showed that if physiological NaCl solution was used in the preparation of the epithelialcells as is usually done cytochrome c concentration in the intestinal mitochondria obtained was very low (Cyt aa_3 Cyt cc = 1 0 76) This indicates that 0 9% NaCl can extract some components from the intestinal cells and is not suitable for isolation of intestinal epithelial cells for studies on energy metabolism

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D 9

Effect of Facial Nerve Stimulation on the Blood Flow of the Eye and Tongue

By JOHAN STJERNSCHANTZ and ANDERS BILL *Institute of Physiology and Medical Biophysics Biomedical Centre University of Uppsala Sweden*

The effect of intracranial stimulation of the seventh cranial nerve (n facialis-n intermedius) on the blood flow of the eye and the tongue was studied in rabbits The nerve was electrically stimulated with a stereotaxic technique: pulse intensity 6–14 V duration 1 msec, and frequency 40–50 Hz. Regional blood flow in the eye and the tongue was measured with radioactively labelled microspheres.

Stimulation caused a 12±39% (n=18) (p<0 001) increase in choroidal blood flow on the stimulated side as compared to the control side Flow rates in the ciliary processes and the iris increased by 53±19% (n=18) (p<0 02) and 1 1±.5% (n=18) (p<0 001) respectively Retinal blood flow also tended to increase during stimulation The blood flow of the mucous membrane of the anterior part of the tongue increased by 210±64% (n=17) (p<0 01) on the stimulated side The blood flow of the corresponding muscle layer of the tongue also increased but with a great variability in the results The duration of the vasodilation of the eye after a period of stimulation was 30–60 min while in the tongue it was about 10 min. Neither the increase in blood flow of the eye nor that of the tongue could adequately be abolished by cholinergic blockade with biperiden (5–10 mg/kg b w) or by ganglionic blockade with hexamethonium (50–100 mg/kg b w) Transsection of the trigeminal nerve prior to stimulation of the facial nerve did not prevent the vasodilation

The phenomenon of atropine-resistant vasodilation in the tongue as a result of corda tympani stimulation is well-known and recent studies by Hellekant (1977) indicate that it is due mainly to activation of nerves originating in the CNS and not in the geniculate ganglion, that is to nerves which seem to be efferent. It is also known that close arterial injection of acetylcholine causes marked but short lasting vasodilation in the choroid in rabbits (Bill 1962)

The efferent autonomic nerves known so far in the intermedio-facial nerve are cholinergic and have synapses in the pterygopalatine or submandibular ganglia Such efferent nerves cannot explain the present results The results rather indicate that the intracranial part of the intermedio-facial nerve contains unconventional efferent or afferent fibres that on stimulation release a vasodilating substance in the eye and the tongue that is not acetylcholine

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D 10

Turnover of Noradrenaline Dopamine and 5-Hydroxytryptamine in the CNS under the Influence of 4-Aminopyridine

By Nils-Erik Andén and Stefan Leander. *Department of Pharmacology Universities of Goteborg and Lund S ede*

4-Aminopyridine (4-AP) enhances nerve impulse evoked release of both acetylcholine and noradrenaline from peripheral nerves (Leander et al. 1977 Lundh et al 1977) 4-AP also

TABLE I Protein yield cytochrome concentrations succinate dehydrogenase activity and oxygen consumption using succinate as a substrate in mitochondria of intestinal epithelial cells. Values are mean values ± S E. from 1 –14 determinations

Protein yield mg/100 g body wt	7 2 ±0.52
Cytochrome aa_3, nmol/mg protein	0.29±0 02
Cytochrome b nmol/mg protein	0.28±0 02
Cytochromes c+c nmol/mg protein	0.38±0 03
Cytochrome aa_3, Cytochromes c+c	1 1.3
Succinate dehydrogenase μmol/min/mg protein	
Actual	0.22±0 02
Maximal	0.55±0 03
Oxygen consumption natoms/min/mg protein	
State 4	18 9±5.3
State 3	25.3±2.1
Respiratory control ratio State 3/State 4	1 4±0.06

In the present preliminary work conditions of the isolation of rat intestinal epithelial mitochondria and their general properties were determined Intestinal epithelial cells of Sprague Dawley rats were separated principally according to Harrison and Webster (1969) but using 0 3 M mannitol—10 mM Tris—5 mM EDTA—3 mM MgCl solution pH 7 4 The cells were sedimented and intestinal mitochondria were isolated using the same medium Mitochondrial cytochromes were determined in 1 M sucrose at liquid nitrogen temperature (Wilson 1967) Absorption coefficients of De Jonge and Hülsman (1973) were used for calculating the cytochrome concentrations Both actual and maximum activity of succinate dehydrogenase were determined by the method of King (1967) by using substrate activation (Kimura *et al* 1967) Mitochondrial oxygen consumption was determined using a Clark type oxygen electrode The results are represented in Table I

The present results also showed that if physiological NaCl solution was used in the preparation of the epithelialcells as is usually done cytochrome c concentration in the intestinal mitochondria obtained was very low (Cyt aa Cyt cc = 1 0 76) This indicates that 0 9% NaCl can extract some components from the intestinal cells and is not suitable for isolation of intestinal epithelial cells for studies on energy metabolism

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D 9

Effect of Facial Nerve Stimulation on the Blood Flow of the Eye and Tongue

By JOHAN STJERNSCHANTZ and ANDERS BILL. *Institute of Physiology and Medical Biophysics Biomedical Centre University of Uppsala Sweden*

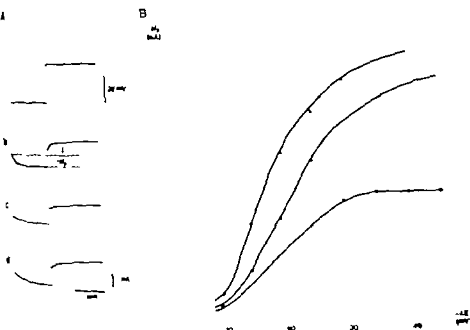


Fig. 1 A. recordings of membrane polarisation (trace a) and membrane current in slowly (trace b) and in fast (traces c and d) adapting lobster stretch receptor neurone. Traces b and c were obtained in 5 mM, and trace d in 10 mM extracellular K. B. relationship between membrane hyperpolarisation ($-\Delta V$) and pump current decrease ($-\Delta I_p$, as defined in panel A) in a slowly adapting cell (triangles) and in fast adapting cell (circles) in 5 mM (open symbols), and in 10 mM (filled symbols) extracellular K.

In lobster stretch receptor neurones slow and reversible decreases in outward membrane current can be produced by hyperpolarising voltage clamp pulses (Fig. 1 A). In comparable conditions these decreases are always much larger in slowly than in fast adapting cells. In both cells they are diminished by all procedures which lead to a reduction in Na–K pump activity. Such procedures include cooling, treatment with ouabain or 2,4-dinitrophenol, and lowering the perineuronal K concentration. Conversely, the voltage dependent current decreases are enhanced by pump stimulating procedures such as warming or increasing the extracellular K concentration (Fig. 1 B). For these reasons they are regarded as evidence of the Na–K pump and its pump current being voltage dependent in hyperpolarising potential regions.

Possibly this voltage dependence is mediated by K whose concentration near the pump sites may vary with voltage dependent variations in transmembrane K leakage on condition that a diffusion barrier existed between the perineuronal space and the surrounding fluid compartment. In such circumstances, polarisation induced reversal of the transmembrane K leakage might lower the perineuronal K concentration below normal levels and thus produce a reduction in Na–K pump activity.

produces CNS-stimulation (Jankowska et al 1977) by enhancing the release of various transmitters Therefore it was of interest to investigate the effect of 4-AP on the turnover of the central monoamines noradrenaline (NA) dopamine (DA) and 5-hydroxytryptamine (5-HT)

The experiments were done on intact or spinalized male Sprague Dawley rats The amine concentrations in the brain and in the spinal cord were determined fluorometrically after cation exchange chromatography 4-AP was given at a dose of 3 mg/kg i p which is the highest subconvulsive dose

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D 11

Polarisation Dependence of the Na–K Pump in Lobster Stretch Receptor Neurones

By C G Å EDMAN and W GRAMPP *Department of Physiology and Biophysics University of Lund Sweden*

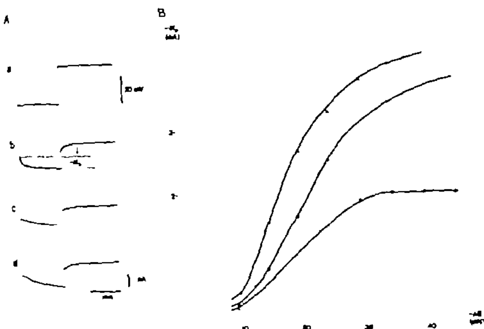


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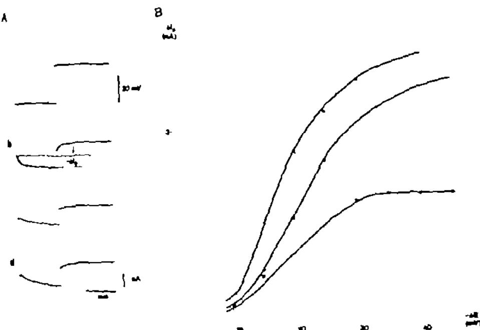


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D II

Polarisation Dependence of the Na–K Pump in Lobster Stretch Receptor Neurones

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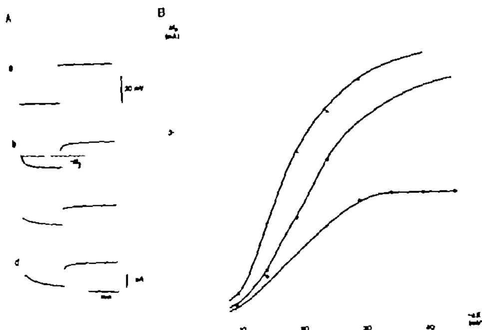


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D 11

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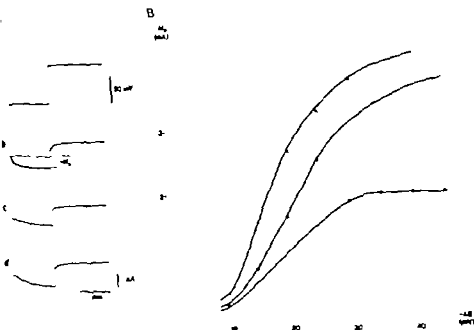


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D 11

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D 14

Uterine Secretomotor Innervation

By M Hammarström and N O Sjöstrand *Department of Physiology Karolinska Institutet Stockholm Sweden*

Nerves to the endometrial linings have been noted in several morfological studies. No functional study seems to have been made Here data indicating a cholinergic secretomotor nervous supply to the endometrium are presented The estimation of uterine secretion was based on the fact that uterine mucus contains carbohydrates

Free-dissected uterus from guinea-pigs treated with estrogen and progesterone for 10–14 days were divided through the cervix, each horn was everted and mounted on an electrode (Sjöstrand and Swedin, 1970) and put into an organ bath containing Tyrode Some animals were denervated 10–12 days before experiment. Some experiments were performed with the pelvic or the hypogastric nerve attached to the organ After a stabilization period of 80 min. in ordinary Tyrode the organ was exposed to five sampling periods of 10 min in glucosefree Tyrode in order not to disturb the carbohydrate determination Each sampling period was interluded by 20 min. of stabilization and nourishment.

The first third and fifth period was used to determine resting level secretion, during the second and fourth period the organ was exposed to nerve field stimulation (0.5 Hz 30 V 0 1 ms) to hypogastric or pelvic nerve stimulation (5 Hz 30 V 0 1 ms) and/or to drugs

The carbohydrate amount of the collected fluid (25 ml) was analyzed according to Dische and Popper (1926) and calculated as glucose

Field stimulation produced in increase by 71%±6 6 in carbohydrate secretion This increase was abolished by TTX ($3\times10^{-}$ M) or scopolamine (1.3×10^{-6} M) Sotalol (1.5×10^{-5} M) and phentolamine ($1\ 6\times10^{-6}$ M) did not affect the answer Pelvic nerve and cervicouterine connexion denervation did not influence the increase in carbohydrate secretion due to field stimulation. However hypogastric denervation reduced the answer to one third and para-cervical ganglion denervation almost abolished the answer Hypogastric nerve stimulation resulted in an increased secretion by 40%±3.2 whilst pelvic nerve stimulation apparently not affect the carbohydrate secretion (8 7%±8 7) Carbachol increased the answer with a sharp threshold at 5.5×10^{-8} M and maximum reached at 5.5×10^{-7} M

Thus we suggest a cholinergic sympathetic secretomotor supply to the guinea-pig endometrium the hypogastric nerve being the main path and some fibers relaying in the para-cervical ganglia

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The fact that at a given external K concentration the polarisation dependent Na–K pump inhibition is smaller in the fast than in the slowly adapting cell corresponds with the observation of a lesser pump activity in the former cell This in turn may be related to the possibility that because of a smaller transmembrane K leakage the perineuronal K concentration was lower in the fast than in the slowly adapting cell

D 12

Tonic Contraction in Chemically Skinned Fibres from the Anterior Byssus Retractor of the Mollusc *Mytilus edulis* (ABRM)

By F Cornelius *Biophysics Institute Aarhus University Denmark*

Dependent on the method of stimulation the living ABRM can produce two kinds of contractions Repetitive electrical shocks cause a phasic contraction which is characterised by a high relaxation rate Acetylcholine (ACh) induces a tonic contraction. In the presence of ACh the developed tension remains nearly constant and the muscle is active judged from quick release experiments A third state ('catch') appears when *ACH is* washed out Relaxation is much slower than for phasic contraction and active state disappears progressively with time (Jewell 1959) Distinction between the two types of contractions and the catch state can thus be made by the time course of relaxation and the degree of active state

In ABRM fibre bundle preparations treated with the nonionic detergent saponin (Endo et al 1977) contraction is initiated when the fibres are exposed to activation solution containing a high free Ca^{2+} concentration (pCa=4 5) The muscle can produce maximal tension over a period of several hours without decay of the active state When a relaxation solution (pCa >8) is applied the muscle relaxes extremely slowly and the active state declines with time The rate of relaxation can be greatly increased by applying serotonin or c AMP The chemically skinned fibres can thus be made to produce contractions with the same characteristics as for tonic contraction and catch state in the living ABRM

The response of the preparation to varying Ca^{2+} concentration was found to be dependent on the free Mg^{2+} concentration At very high Ca^{2+} concentrations (pCa <4) the force generating capacity of the muscle was depressed This depressant effect could however be eliminated by raising the free Mg^{2+} concentration At optimal Ca^{2+} concentration an increase in the Mg^{2+} concentration caused an increase in the rate of relaxation

The results suggest that Mg^{2+} may play a role in the regulation of the catch state

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D 13 not printed

saturation kinetics is different in the three groups of animals. From a double reciprocal plot a K_t of 4 mM 11 mM and 22 mM was calculated for the group with low—normal—and high cerebral glucose concentration respectively

A model of the blood-brain barrier with one Michaelis Menten membrane in the capillary wall was fitted to the data. A reasonable fit was obtained with small variations of K_t. A model constituted of two Michaelis-Menten membranes in series symbolizing the luminal and the contraluminal endothelial cell membrane gave an even better fit with constant K_t values. Thus the apparent increase of K_t with increasing cerebral glucose concentrations seems to reflect a methodological problem rather than a real change of the transport mechanism. This conclusion has a bearing on many experiments on glucose transport in the brain (Lund-Andersen 1979)

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D 16

Contracture-Potentiating Effects of the SH Blocking Para-Mercuribenzoates

By A. RØED, *Department of Physiology and Biochemistry, Dental Faculty, University of Oslo, Norway*

The mercuribenzoate sulfhydryl-blocking agents pClMB pClNBS and pOHMB (100 μM) cause no contractures in the rat diaphragm preparation by themselves but potentiate the contracture response caused by other agents. These effects are described and their dependency on the myotonia caused by the SH-blocking agents (Røed 1976) and the significance of SH binding for the effects are investigated

The biphasic caffeine (10 mM) contracture was increased in amplitude upon pOGMB (100 μM) pretreatment. The first phase increased from 12.4±3.5 per cent to 42.8±14.5 per cent of the maximal twitch amplitude prior to drug addition. This maximum tension appeared after 7–9 min in the pOHMB-treated preparation while in the control preparation it appeared after 25–30 min.

The phasic depolarization contracture caused by KCl (100 mM) was increased in amplitude from 70.7±15.5 per cent to 125.2±14.8 per cent after prior exposure to pOHMB (100 μM). Moreover the time of maximal amplitude was shortened from about 20 sec to 5–6 sec by pretreatment with pOHMB

Some of the membrane-stabilizing drugs with local anaesthetic activity like propranolol quinine and dibucaine caused a biphasic contracture in the 1 mM concentration. In the control preparation, the contracture was preceded by a delay of 2–3 min which was completely obliterated by pOHMB pretreatment. The amplitude of the first phase of the

D 15

Glucose Transport Across the Blood-Brain Barrier: On the Apparent Effect of the Cerebral Glucose Concentration upon the Transport Affinity Constant

By H LUND-ANDERSEN and O CHRISTENSEN *Institute of Medical Physiology Dept A The Panum Institute and Department of Opthalmology Rigshospitalet University of Copenhagen Denmark*

The transport of D-glucose from blood to brain across the blood-brain barrier exhibits Michaelis Menten saturation kinetics (Crone 1965) It has been shown by Betz et al. (1975) that the transport affinity content K_t apparently increases from 8 to 37 mM when the cerebral glucose concentration increases from 2 to 12 mM However in their type of non-steady state experiments the glucose concentration at the carrier sites on the luminal and on the contraluminal endothel cell membrane might be underestimated when glucose concentration in the bolus was lower than that in the brain because glucose passes out of the brain through the endothelial cells into the blood This may explain the apparent increase of K with increasing cerebral glucose concentrations

This hypothesis was tested by fitting models of the blood brain barrier transport to kinetic data obtained on rats with a cerebral glucose concentration which was low normal and high The data were obtained by aid of the non-steady state single injection technique developed by Oldendorf (1971) The results are given in Fig 1 It appears that the

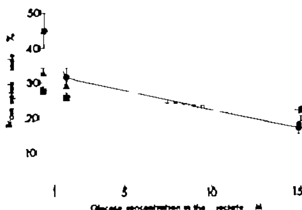


Fig. 1 Transport of the non metabolizable glucose analogue ^{14}C 3-O-methylglucose across the blood-brain barrier relative to the transport of $^{3}H_2O$ (brain uptake index BUI) after injection of an isotope containing bolus in the common carotid artery (Oldendorf 1971) Decapitation was performed 5 sec after injection The inhibitory effect of increasing glucose concentrations (0 17 mM 1.5 mM and 15 mM) in the injectate on the transport of labelled 3-O-methyl-glucose was studied on three groups of rats which prior to injection were in steady state at different plasma and brain glucose concentrations: (●–●) indicates plasma glucose of 1 mM and brain glucose of about 0 mM (▲–▲) indicates plasma glucose of 7 mM and brain glucose of about mM and (■–■) indicates plasma glucose of 35 mM and brain glucose of about 10 mM Each point is the mean of 3–6 experiments with S E.M indicated by vertical bars

permeability was negligible indicating that there was no significant contribution of choroidal blood to the plexus.

In anaesthetized pigs the following a-v-differences in mMol/l were observed for the retina and choroid respectively (mean ± SE and number of experiments): Glucose 0.49±0.06 (13) 0.14±0.06 (10) Oxygen 2.50±0.24 (12) 0.15±0.03 (11) Lactate—0.25±0.10 (8)—0.29±0.11 (8) All differences were significant at the $p<0.05$ level or less. Studies with the labelled microsphere technique provided data for retinal and choroidal blood flow in anaesthetized pigs 64 and 1732 mg/min respectively. Then if the metabolism of the choroid is neglected and the admixture of blood from the anterior uvea is assumed to cause no major change in venous O_2, glucose and lactate concentrations it can be estimated that the contribution to retinal metabolism from the retinal vessels is 161 and 32 nMol/min and from the choroid 260 and 242 nMol/min of oxygen and glucose respectively. The oxygen provided can only account for complete oxidation of 26% of the glucose. A major part of the glucose thus seems to be metabolized to lactate. The values for glucose and oxygen consumption and the lactate production found in the present study agree well with the results obtained in *in vitro* studies on rats (Reading and Sorsby 1962)

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D 18

Sympathetic Effects on Cerebral Circulation in Acute Stress

By M. BEAUSANG-LINDER and J LINDER. *Department of Physiology and Medical Biophysics Uppsala Sweden*

The blood vessels of the brain have a rather rich supply of sympathetic nerves. The physiological role of these nerves has been debated upon for a long time (Lassen 1974). During normotension electrical stimulation of the cervical sympathetic chain has practically no effect on cerebral blood flow as determined with labelled microspheres (Alm and Bill 1973). In acute hypertension in anaesthetized animals sympathetic stimulation has been shown to have significant effects (Bill and Linder 1976). Stimulation helps to prevent forced vasodilatation, overperfusion with blood and breakdown of the blood-brain barrier.

The purpose of these experiments was to study the physiological importance of the sympathetics in acute stress. The experiments were performed in awake SHR rats. The animals were cannulated and unilaterally sympathectomized the day before the experiment. The acute stress and blood pressure rise was caused by forcing the animals to swim in cold water. The blood-brain barrier was studied using 125-iodinized albumin. As a macroscopic tracer Evans blue was used. A rise in MABP of 40–105 cm H_2O was induced. The pressure rise tended to cause a break down of the blood-brain and blood-aqueous barriers, effects that seemed to be more marked on the sympathectomized side

propranolol contracture was 40 8±8 4 per cent in the control preparation and 48.4±15 7 per cent after pOHMB pretreatment. This increase was not significant.

The membrane stabilizer diphenylhydantoin which lacks local anaesthetic activity caused a monophasic contracture with a maximal amplitude of 10 0±3.2 per cent within 2 min in the pOHMB-treated preparation in 1 mM concentration This contracture was not *observed in the control preparation*

Myotonia appeared upon pretreatment with pOHMB However the contracture potentiating effect was independent upon the presence of myotonia. Selective block of the myotonic response with a local anaesthetic did not reduce the contracture-potentiating ability and experiments on the slow twitch rat soleus muscle showed high contracture-*potentiating ability combined with no myotonic response*

All the contracture-potentiating effects of pOHMB were dependent on the SH-binding properties of pOHMB because these effects were not observed when the preparations were pretreated with dithiothreitol (100 μM) which keeps the SH groups in the reduced state (Cleland 1964)

On the other hand contracture potentiation was not a general result of SH inhibition. It was not observed after pretreatment with N-ethylmaleimide another SH-blocking agent

In conclusion it is suggested that the mercuribenzoates interact with SH groups and thereby indirectly increase the availability of the Ca compartments being released by the contracture producing agents

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D 17

Retinal and Choroidal Contribution to Retinal Nutrition

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The retina is supplied *from two sources the retinal capillaries and the choroidal capillaries* The retinal metabolism under *in vivo* conditions can be studied by determining a-v-differences for substrates and metabolites venous blood being sampled both from the choroid and the retina. Choroidal blood with a moderate admixture of blood from the anterior uvea can be obtained by cannulating a vortex vein Retinal venous blood is difficult to obtain *due to the inaccessibility* of the retinal veins A technique was developed in pigs that permits cannulation of a venous plexus around the optic nerve By aid of silicone rubber casts this plexus was shown to drain retinal blood Previous studies with the single injection technique (Törnquist 1977) have shown a considerable *permeability* to sodium in the choroid This technique applied to the retina showed that the sodium

vascular disturbance the perfusion of the nerve head being reduced to levels causing undernutrition of the nervous tissue The arrangement of the vessels supplying the distal part of the nerve is complex branches from the central retinal artery and short posterior ciliary arteries supplying poorly defined regions It has been suggested that elevation of the intraocular pressure causes a redistribution of the nerve head blood flow to extraocular regions and also that autoregulation of the optic nerve blood flow might be poor compared to that in the retina.

In monkeys the arrangement of the vascular supply to the optic nerve is similar to that in man and model glaucomas can be produced We have now used unlabelled 8–10 um microspheres to study the effects of acute and subacute elevations of the intraocular pressure on the blood flow in the different regions of the peripheral part of the optic nerve The regions investigated were the prelaminar part of the nerve the laminar part and the first, second and third millimeters of the nerve behind the lamina. The central retina was also investigated. One eye had its normal eye pressure and the other had its pressure stabilized at a higher level to reduce the perfusion pressure defined as the mean arterial pressure minus the intraocular pressure Unlabelled microspheres were injected into the left heart ventricle The eyes were frozen and sectioned and the number of spheres in the different regions counted. The results indicate that there is efficient autoregulation of the blood flow in the retina and optic nerve head within a wide range of perfusion pressures Laminar flow is not stopped at pressures stopping flow in the eye and close laminar flow behind the lamina is increased at high intraocular pressures most probably due to diffusion of metabolites from the prelaminar region through the lamina. After a period with high intraocular pressure reduction in eye pressure results in reactive hyperemia in the retina, and in the prelaminar and laminar parts of the nerve The results do not offer any simple explanation for the development of optic nerve damage in glaucoma.

D 21

The Time Course of Adaptation in the Retinular Cells of the Fly

By I. MORING M. JARVILEHTO and K. MORING *Department of Physiology University of Oulu Finland*

The sensitivity of photoreceptor cells is controlled by photomechanical, photochemical and neural mechanisms. The amount of energy required to activate the photochemical mechanisms is relatively high and photomechanical reactions are known to be slow At low levels of light energy the neural mechanism is responsible for fast changes of light sensitivity in photoreceptor cells (for review see Goldsmith 1975) This mechanism is believed to affect the chain of chemical events leading from the absorption of light quanta to the electrical response of the cell. Another possibility for sensitivity control is an inhibitory feedback. There is evidence of both in vertebrate and invertebrate eyes

In the case of inhibitory feedback alone as an adapting mechanism, the onset of sensitivity reduction would be delayed. On the other hand a pure membrane mechanism could lead to the reduction of sensitivity already during the latency time of the photoresponse

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D 19

A Method to Determine the Rate of Aqueous Humor Formation Some Preliminary Results

By G SPERBER and A BILL *Department of Physiology and Medical Biophysics Uppsala Sweden*

The aqueous humor produced by the ciliary processes flows from the posterior chamber into the anterior chamber and is drained in the iridocorneal angle in such a way that it cannot be collected quantitatively Indirect studies in the rabbit have indicated that changes in the pH of the blood produced by infusion of sodium bicarbonate or hydrochloric acid have marked effects on aqueous humor formation Alkalosis stimulated formation acidosis inhibited (Krupin et al 1977)

We have developed an isotope dilution procedure for direct studies on aqueous humor production in monkeys on-line analysis of dilution data making it possible to determine aqueous humor formation rate every few minutes at normal intraocular pressures Push-pull-coupled syringes are used to perfuse the anterior chamber with a solution of radioactive albumin at a rate of 1 6 μl/min An external circuit and a circulation pump are used to mix the fluid in the anterior chamber and pump fluid through a gamma-detector Counting rates are determined and treated by a minicomputer and flow rates calculated every second minute *The flow rates calculated 1–2 μl/min are fairly steady over hours and agree well* with known data for monkeys Infusion of sodium bicarbonate and hydrochloric acid have smaller effects in monkeys than in rabbits Indomethacin 2–20 mg/kg b w has no effect on the normal flow rate but seems to prevent an irritative response otherwise sometimes appearing after 4–5 hours of perfusion

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D 20

Optic Nerve Blood Flow Measured with Microspheres. Effects of Ocular Hypertension

By CHRISTOFFER GEIJER and ANDERS BILL. *Department of Physiology and Medical Biophysics Uppsala Sweden*

In glaucoma there is usually a high pressure in the eye and it has been assumed that the degeneration of the optic nerve that is characteristic of the disease is a consequence of a

[illegible] I Effect of insulin and adrenaline of ^{22}Na-efflux, ^{42}K-influx and E_M in rat soleus muscle For experimental [illegible], see Clausen & Flatman, 1977 All results given ± S.E. with the number of observations [in] parantheses ([for] E_M the number of fibres penetrated [and] the number of muscles investigated).

	^{22}Na-efflux (fraction of ^{22}Na lost per min)	p	^{42}K-influx (µmoles/g/min)	p	ΔE (mV) (increase in E_M in relation To untreated control muscles)	p	
[illegible]	0 045±0 003 (3)	<0.005	0 6_±0.02 (8)	<0 005			
[illegible]	0 081+0 005 (3)		0 74±0 02 (8)		3.5±0 4 (224/6)	<0 001	
[illegible]	0 106+0 003 (3)	>0 10	0.80±0 03 (8)	<0.01	7 7_0 3 (240/6)	<0.001	<0 05
[illegible]	0 110±0 003 (3)		0 90±0 02 (8)		6 6 0 4 (230/6)	<0 001	

both hormones indicating it is related to a stimulation of active Na–K transport The effect of adrenaline and insulin were partially additive on Na–K transport whether insulin was added before after or simultaneously with (Table I) adrenal ne However the hyperpolarization produced by insul n and adrenaline combined (insulin added before adrenaline) was no larger than that produced by adrenaline alone Indeed the hyperpolarization produced by adrenaline is reduced by the later addition of insulin.

Also in vivo (pentobarbitone anaesthetized _50 g rats) insulin and adrenaline (or salbutamol) individually increased E_M in combination their actions were additive

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D 23

Role of Physical Fitness in Lipolytic Activities of Human Postheparin plasma

By P Peltonen J Marniemi I Vuori and E Hietanen *The Rehabilitation Research Centre of the Social Insurance Institution Turku and Department of Physiology University of Kuopio Finland*

The triglyceride lipase activities released into the blood circulation by heparin injection have been studied widely in certai disordered states of fat metabolism The effect of physical exercise on human lipolytic enzymes i still obscure In laboratory animals endurance training has increased significantly lipoprotein lipase activity of the skeletal

To reveal which mechanism is responsible for light adaptation we measured the time course of adaptation by using an adapting flash and a test flash both with a duration of 10 µs and an estimated maximum quantum content of 10^{12} The change of sensitivity was measured between 0 and 300 ms after the adapting flash The latencies of the photoresponses were between 3 and 7 ms and the duration of the response up to ?0 ms. The amplitudes (maximally up to 40 mV) of the responses to the test flashes were converted to effective intensities the relationships of these and the effective intensity of a nonadapted control response were used to describe the sensitivity of the cell The effect of nonlinear summation of the responses to adapting and test flashes on sensitivity was eliminated by using the V logI functions of the cells The sensitivity of the receptor cells began to decrease immedeately after the adapting flash even before the electrical potential response was produced This indicates that sensitivity is controlled at the beginning of the chain leading to the photoresponse and the mechanism does not require a change of membrane potential The decrease of sensitivity continued to a least 15 ms and turned to dark recovery between 25 and 50 ms The recovery was complete at approximately 300 ms in our experimental conditions The nature of the mechanism is probably a release of an inhibitory substance (Ca ?) at the beginning of the chain leading to the photoresponse Light adaptation can further be reinforced by inhibitory feedback which can control the sensitivity only after the photoresponse is produced

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D 22

Combined Effect of Adrenaline and Insulin on Active Electrogenic Na–K Transport in Rat Soleus Muscle

By J A FLATMAN and T CLAUSEN *Institute of Physiology University of Aarhus Denmark*

In the isolated soleus muscle of the rat adrenaline has been shown to stimulate the active electrogenic Na–K transport (Clausen & Flatman 1977) Insulin likewise stimulates active Na–K transport (Clausen & Kohn 1977) and in rat EDL muscle the membrane potential (E_M) is increased (Zierler 197?) We have now compared the actions of these agents alone and combined in both "in vitro" and "in vivo" experiments

Na–K transport and E_M were measured in soleus muscles from 60–70 g fed rats In order to obtain more stable recordings of E_M several series of measurements were performed on rats weighing c 250 g

Supramaximal concentrations of either insulin or adrenaline stimulate ^{22}Na efflux ^{42}K influx and increase E_M (Table I) The effects of insulin were somewhat less pronounced and slower in onset than those of adrenaline Ouabain blocked the hyperpolarizing effect of

D 24

Bradycardia in Endurance Trained Men Due to Non-Autonomic Factors

By STEVEN LEWIS, PRAM GAD EVA NYLANDER and NILS-HOLGER ARESKOG *August Krogh Institute University of Copenhagen Denmark and Dept of Clinical Physiology Regionsjukhuset Linköping University S eden*

The relative bradycardia of endurance training in man is to some extent attributable to changes in the autonomic nervous system (Ekblom et al 1973) However a lower resting heart rate (HR) after combined vagal and beta-adrenergic blockade has been found in endurance trained rats than in untrained rats (Hughson et al. 1976) In addition factors which establish the intrinsic HR in these animals seem to limit maximum exercise HR (Hughson et al., 1976)

In the present study 8 healthy sedentary men (SED) (age 26±4 yr mean±SD) and 8 long distance competition bicyclists (ATH) (age 21± yr) were compared at rest and during exercise on a bicycle ergometer at workloads corresponding to approximately 50 75 and 100% of maximal oxygen uptake ($VO_{2\ max}$) ATH had trained 400–700 km/week for 3-11 years prior to the study Testing was performed under normal conditions and after combined vagal blockade with atropine (0 04 mg/kg b w) and beta-adrenergic blockade with propanolol (0.25 mg/kg b w) or metoprolol (0.5 mg/kg b w) injected intravenously In nearly all comparisons very similar results were obtained after using metoprolol or propanolol. The exercise tests were carried out in ascending order of intensity and an additional dose of 0 05 mg/kg propanolol or 0 1 mg/kg metoprolol was injected prior to the 75% max. load.

$VO_{2\ max}$ was 45±6 (SED) and 76±5 (ATH) ml/kg min after blockade (AB) $VO_{2\ max}$ was reduced by approximately 20% in ATH and 10% in SED Maximal HR was approximately 40 beats/min lower in both groups after AB At rest and during exercise at 50 75 and 100% $VO_{2\ max}$ under normal conditions and after AB HR was lower in ATH than in SED ($p<0.05$) The mean HR difference between SED and ATH was very similar in magnitude (approximately 13 beats/min) at rest and at all levels of exercise under normal conditions and after AB

These data indicate that prolonged intense endurance training may result in non-autonomic changes which reduce HR at rest and during exercise at any given level of relative cardiovascular stress One non-autonomic factor which may play a role in the bradycardia is cardiac enlargement. Heart volume estimated from roentgenograms at rest was 420+83 and 538±78 ml/BSA in SED and ATH respectively ($p<0.05$) and when the data from the groups were combined (N=16) heart volume and resting HR were inversely related (0 64)

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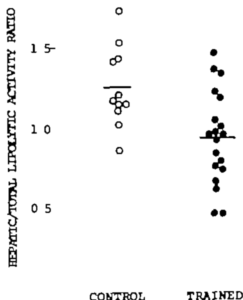


Fig. 1 Individual values of postheparin plasma hepatic/total lipolytic activity ratios in male non-obese trained (●) and nontrained control (○) subjects. The means are indicated by horizontal lines. The training consisted of skiing running swimming or other heavy exercise being repeated regularly several times a week. The significance of the difference between the means of the groups (calculated by Student's t-test) is: P<0 005

muscle (Borensztajn et al 1975) but the effect of short term exercise in man was negligible (Marniemi et al 1978)

For the determination of the lipolytic activity the radiochemical method with ^{14}C-trioleín as substrate as described by Huttunen et al (1975) was modified The differentiation between hepatic and extra hepatic (lipoprotein) lipases was carried out utilizing the inhibition of extra-hepatic enzyme by 1 0 mol/l NaCl without any specific antiserum The activity of hepatic lipase at this salt concentration is considerably higher than at 0 1 mol/l NaCl (Huttunen et al 1975) thus causing the hepatic enzyme/total activity ratio to be higher than one in some cases Heparin (50 IU/kg) was administered intravenously into test subjects 10 min before the plasma sample was taken

A positive correlation was found between the relative weight of the subjects and lipolytic activity On the other hand a negative correlation was obtained between the serum triglyceride concentration and enzyme activity The hepatic/total lipolytic activity ratio in postheparin plasma appeared to be decreased in actively training subjects when compared to non training controls (fig 1) This difference is partly due to the decrease in the hepatic activity observed and partly perhaps due to increase in muscle lipoprotein lipase The final clarification of this phenomenon in man awaits for the analysis of tissue samples and the plasma enzyme determination with the specific immunochemical method These studies are in our next program

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D 26

The Effect of Fasting on the Response to Exercise of Catecholamines in Plasma

By H. GALBO and N. J. CHRISTENSEN. *Institute of Medical Physiology B, University of Copenhagen, and Department of Internal Medicine and Endocrinology, Herlev Hospital, Denmark*

Fasting profoundly changes the endocrine and metabolic state of the organism. We have now for the first time studied the sympathoadrenomedullary response to exercise after prolonged fasting.

6 healthy men (25±2 yr (mean ± SE); V_{O_2} max. 55±1 ml kg⁻¹ min⁻¹) had blood drawn from the subclavian vein at rest and at the end of two successive treadmill runs lasting 10 and 5 min and requiring 50 and 100% of V_{O_2} max, respectively. This was done after an overnight fast (C-expts.) and furthermore three times separated by 30 min rest intervals (D, E and F-expts.) after 59 h fasting. During the submaximal (0.75 g min⁻¹) and maximal (1.16 g min⁻¹) exercise in E-expts. a 30% (g 100 ml⁻¹) glucose solution was infused.

Fasting increased heart rate at rest, submaximal and maximal exercise (88±2, 163±, 192±2 beats min⁻¹ (D-expts.) vs. 77±4, 151±5, 187±3 (C-expts.)). Also noradrenaline values were significantly higher in D- than in C-expts. (0.65±0.09, 1.52±0.17, 6.64±0.75 ng ml⁻¹ vs. 0.30±0.06, 0.87±0.10, 3.41±0.56) and adrenaline were higher during work (0.15±0.02, 1.45±0.48 ng ml⁻¹ (D-expts.) vs. 0.12±0.01, 0.42±0.10 (C-expts.)). FFA, glycerol and hematocrit (45.9±1.1, 47.4±0.8, 48.0±0.8% (D-expts.) vs. 43.5±0.7, 45.1±0.7, 47.1±0.7 (C-expts.)) were higher and plasma glucose concentrations lower (75, 74±4, 78±3 mg 100 ml⁻¹ (D-expts.) vs. 94±2, 91±3, 94±3 (C-expts.)) after fasting. During glucose infusion hematocrit values were similar to values in C-expts. and plasma glucose concentrations were higher (157±10, 191±14). At the high workload heart rate (180±1) and noradrenaline (5.25±0.72) were significantly lower in E- than in D-expts., but noradrenaline was at this as well as at the low workload still significantly higher than in C-expts. Also in the final treadmill runs after fasting (F-expts.) the glucose concentrations were higher and hematocrit lower than in D-expts., but noradrenaline and heart rate were higher. Adrenaline concentrations were identical at similar work loads in the three exercise periods after fasting.

In conclusion, fasting markedly enhances the sympathoadrenomedullary response to exercise. The present study indicates that this enhancement is not explainable by changes in plasma glucose concentration or in plasma volume.

D 27

Effect of Adrenal Demedullation on Hepatic Glycogen Depletion and Circulating Catecholamines in Exercising Rats

By E. A. RICHTER, H. GALBO and N. J. CHRISTENSEN. *Institute of Medical Physiology B, University of Copenhagen, and Department of Internal Medicine and Endocrinology, Herlev Hospital, Copenhagen*

D 25

The Effect of Training in Relation to Chronological Age and Developmental Stages in Children 9 to 17 Years of Age

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The increase in dynamic endurance with training has previously been shown to be markedly greater in children aged 12 to 13 years than in both younger and older children (Ikai 1969) This suggests an increased sensitivity to training during puberty

In order to test this hypotesis the above mentioned training experiment was reproduced using 240 children between the age of 9 and 17 years of age The increase in dynamic endurance in the right arm flexors was measured and related to the chronological age and the following three developmental stages The biological age (Greulich and Pyle 1959) and the puberal growth spurt (Helm et al 1971) were predicted from radiographs of the hand and wrist The dental age (Demirjian et al 1973) was obtained from a clinical investigation and radiographs of the teeth

The results in relation to chronological age are not in agreement with the results of Ikai We found that the relative mean increase in the dynamic endurance was 35% for the 13.5 years old boys and 29% for the 12 5 years old girls whereas in all other age groups it was found to be more than 50% for both sexes

When the increase was compared with the different development stages a similar reduced increase was seen for the boys at the dental age of 13 5 years and for the girls at the dental age of 15 5 years A reduced increase was also found at the biological age of 10.5 years for boys while no differences in increase was found for the girls Finally when related to the predicted puberal growth spurt the reduced increase was seen in the group of boys predicted to be half a year from the onset of the puberal growth spurt For the girls it was observed in the groups predicted to be in the middle of this growth spurt and half a year older

Thus our results indicate that the increase in dynamic endurance with training is rather small at a certain chronological age and that this seems to be related to the period connected with the puberal growth spurt The separation of the results in means calculated for each of the two sexes and the earlier onset of the puberal growth spurt seen in girls can to some extent explain the difference between these results and those previously reported

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D 28

Sympathetic Atropine Sensitive Effects on Gastric Motility

D Delbro and B Lisander, *Department of Physiology University of Göteborg Göteborg Sweden*

Efferent splanchnic nerve stimulation can decrease gastric tone by an activation of the adrenergic fibres. At low stimulation frequencies however atropine sensitive augmentatory responses may occur (Thomas and Baldwin 1968) and it was considered of interest to further analyse these less studied excitatory effects

Experiments were performed on chloralosed adrenalectomized cats paralysed with gallamine and artificially ventilated. Gastric motility was recorded by a balloon, introduced via the oesophagus.

Efferent stimulation of the cut greater splanchnic nerves could either increase or decrease gastric tone The excitatory responses occurred at higher stimulation intensities than the inhibitory ones but were as a rule observed at lower stimulation frequencies only (1–4 Hz) They were not prevented by bilateral cervical vagotomy hexamethonium or guanethidine (both at 5 mg/kg) but could be completely abolished by atropine (0 1–0.5 mg/kg). Guanethidine and/or hexamethonium reversed inhibitory responses to excitatory ones and the latter then reached an optimum at 6–8 Hz or above

Similar excitatory responses with the same pharmacological characteristics could be elicited when the thoracic sympathetic trunk was stimulated up to the level of the 7th thoracic vertebra and also from the centrally cut *dorsal* root at Th 10 in agreement with findings by Semba and Hiraoka (1957)

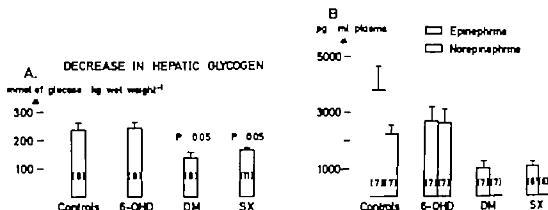
Efferent vagal stimulation in the neck did not evoke any action potentials in the greater splanchnic nerve indicating that the studied excitatory effects were not elicited by vagal fibres running in sympathetic nerves

Heating of a nerve can activate the thinnest afferents of the delta group and C-class (von Euler 1947) Heating of the greater splanchnic nerve caused an increase in gastric motility which could not be prevented by hexamethonium or guanethidine nor by cutting the nerve centrally Atropine however completely abolished these responses

The evidence thus suggests that the excitatory gastric responses to efferent splanchnic nerve stimulation are due to antidromic activation of afferent fibres The functional significance of these effects remains obscure but it is possible that afferent fibres mediate excitatory axonal reflexes within the abdominal cavity

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We have reported (Galbo et al 1978) that in adrenodemedullated rats injected with 6-hydroxydopamine (selectively destroying sympathetic nerve endings) hepatic glycogen depletion during exercise is smaller than in control rats Now we have elucidated if this effect of sympathectomy can be ascribed selectively to the lack of either the adrenal medulla or of the peripheral sympathetic nerve endings

Four groups of rats which 3 weeks previously had been either adrenodemedullated (DM) injected with 6-hydroxydopamine (6-OHD) adrenodemedullated and injected with 6-hydroxydopamine (SX) or sham operated and sham injected controls (C) were used During ether anesthesia a small biopsy of the liver was removed and 15 min afterwards the rats were forced to swim with a tail weight for 100 min Then blood was drawn and a liver sample was collected

In DM and SX rats the declines in hepatic glycogen concentrations (Fig. 1 A) and the increases in blood glucose (Richter et al 1978) were significantly smaller than in 6-OHD and C rats Furthermore in DM and SX-rats epinephrine in plasma was undetectable and the concentrations of norepinephrine were markedly below concentrations in C-rats (Fig. 1 B) 6-OHD did not significantly decrease the concentrations of norepinephrine and epinephrine but halved the norepinephrine/epinephrine ratio (2 38±0 47 ($\bar{X} \pm$ SE) in controls and 1 07±0 09 in 6-OHD (P<0 0?))

It is concluded that in the exercising rat 1) Circulating catecholamines enhance hepatic glycogen breakdown directly or indirectly 2) In contrast to the findings in man (Vendsalu 1960) the major part of plasma norepinephrine is of adrenomedullary origin 3) Plasma epinephrine is entirely of adrenomedullary origin

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VENDSALU A *Acta Physiol Scand* 1960 *49* Suppl. 173

The high affinity binder may represent a specific carrier protein for folate in serum Preliminary studies indicate that a binder with similar characteristics is present in male serum Furthermore folate binding in milk (Hansen *et al* 1977 Hansen *et al* 1978) displays features similar to those of specific folate binding in serum

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D 30

Fate of Receptor Bound Glucagon in Adipocytes

By OLE SONNE and JØRGEN GLIEMANN *Institute of Medical Physiology C The Panum Institute University of Copenhagen Denmark*

The interaction of polypeptide hormones with their receptors (defined as saturable binding sites) has generally been assumed to be a bimolecular reversibel reaction $H + R \rightleftarrows RH$ However in the case of insulin binding to rat adipocytes we have shown (Gliemann & Sonne, 1978) that about half of the ^{125}I-labelled hormone bound to receptors at steady state is degraded and that the radioactivity dissociates from the cells as iodotyrosine.

Receptor-mediated breakdown of polypeptide hormones might be a more general phenomenon and we have therefor studied the fate of receptor bound ^{125}I-glucagon In

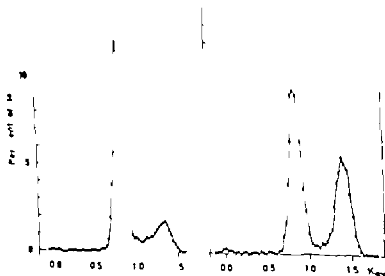


Fig 1 For explanation see text

D 29

High and Low Affinity Binding of Folate in Human Serum

By J HOLM S I HANSEN and J LYNGBYE. *Department of Clinical Chemistry Central Hospital Hillerød Denmark*

The high folate binding activity normally observed in serum during pregnancy prompted the present study on binding of ^3H folate in sera from pregnant women Scatchard analyses of data from Equilibrium dialysis experiments revealed two classes of binding sites a high affinity type K(equilibrium constant)=10 M^{-1} N(maximum bound folate)=0 4 nM and a low affinity type K=10^6 M^{-1} The high affinity folate binder was eluted from sera subjected to anion exchange chromatography (DEAE Sepharose CL) 6B Imidazole buffer of *pH 6 3 with 30 mM NaCl)* The protein was present in tracer amounts and had a molecular weight of 25 000 as determined by Ultrogel AcA—44 gel filtration The low affinity binder (M =70 000) which was eluted from the DEAE Sepharose column following a rise in NaCl to 1 M was identical to albumin

High affinity binding of H folate displays positive cooperativity as indicated by the *downward concavity of the Scatchard plot (Fig 1) Half saturation of binding was achieved* at a folate concentration of 10^{-1} M and saturation at 10 $^{-10}$ M The folate analogue methotrexate is a rather weak inhibitor of folate binding (50% inhibition at a molar methotrexate folate ratio of 100) Inhibition seemed to be of a competitive type since no decrease in maximum bound folate was seen in presence of methotrexate Dissociation of *^3H folate from the high affinity binder was slow and incomplete at pH 7 4* but became rapid and complete at pH 3 5

Low affinity binding of ^3H folate was proportional to the folate concentration within the interval 10^{-10} M to 10^{-7} M Methotrexate had no effect on this binding which was loose and dissociable

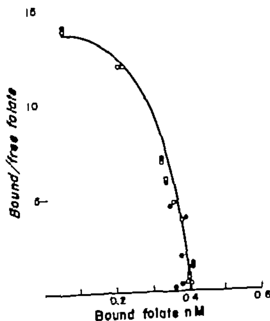


Fig 1 Scatchard plot of folate binding to a high affinity binder in sera from pregnant women. The binder was eluted from sera subjected to DEAE—Sepharose CL-6B ion exchange chromatography (for details cf text). Equilibrium dialysis experiments at 37°C in Tris buffer of pH 7 4 (single determinations) Abscissa bound folate Ordinate bound/free folate

The high affinity binder may represent a specific carrier protein for folate in serum. Preliminary studies indicate that a binder with similar characteristics is present in male serum. Furthermore folate binding in milk (Hansen *et al.* 1977, Hansen *et al.* 1978) displays features similar to those of specific folate binding in serum.

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D 30

Fate of Receptor Bound Glucagon in Adipocytes

By OLE SONNE and JØRGEN GLIEMANN. *Institute of Medical Physiology C, The Panum Institute, University of Copenhagen, Denmark.*

The interaction of polypeptide hormones with their receptors (defined as saturable binding sites) has generally been assumed to be a bimolecular reversibel reaction, $H + R \rightleftharpoons RH$. However, in the case of insulin binding to rat adipocytes we have shown (Gliemann & Sonne, 1978) that about half of the ^{125}I-labelled hormone bound to receptors at steady state is degraded and that the radioactivity dissociates from the cells as iodotyrosine.

Receptor-mediated breakdown of polypeptide hormones might be a more general phenomenon and we have therefor studied the fate of receptor bound ^{125}I-glucagon. In

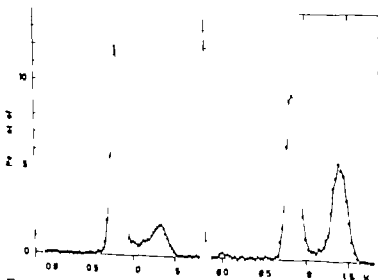


Fig. 1. For explanation see text.

D 29

High and Low Affinity Binding of Folate in Human Serum

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High affinity binding of ^{3}H folate displays positive cooperativity as indicated by the downward concavity of the Scatchard plot (Fig 1) Half saturation of binding was achieved at a folate concentration of 10^{-11} M and saturation at 10^{-9} M The folate analogue methotrexate is a rather weak inhibitor of folate binding (50% inhibition at a molar methotrexate folate ratio of 100) Inhibition seemed to be of a competitive type since no decrease in maximum bound folate was seen in presence of methotrexate Dissociation of ^{3}H folate from the high affinity binder was slow and incomplete at pH 7 4 but became rapid and complete at pH 3 5

Low affinity binding of ^{3}H folate was proportional to the folate concentration within the interval 10^{-10} M to 10^{-7} M Methotrexate had no effect on this binding which was loose and dissociable

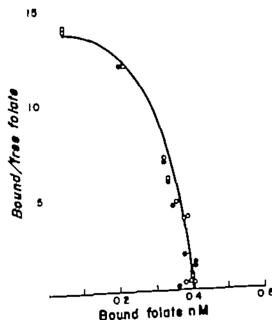


Fig. 1 Scatchard plot of folate binding to a high affinity binder in sera from pregnant women. The binder was eluted from sera subjected to DEAE—Sepharose CL-6B ion exchange chromatography (for details cf text) Equilibrium dialysis experiments at 37°C in Tris buffer of pH 7 4 (single determinations) Abscissa bound folate Ordinate bound/free folate

The cerebral unidirectional extraction of glucose (E) was measured 5 s after a single intracarotid isotonic injection of a flow-limited reference substance and labeled D-glucose according to Oldendorf's method (1970) with or without the presence of 1 mM buffered ammonium acetate in the injectate. In sham-operated rats the presence of ammonia in the injectate caused an increase of E from 13.1% to 18.3% (P<0.05). No change of E was observed in rats with PCA for 4 weeks. In rats with PCA for 8 weeks, the presence of ammonia in the injectate caused a decrease of E from 13.2% to 9.0% (P<0.02). Blood ammonia was increased from the control value of 100 μM to 400 μM in both groups of PCA-rats.

We conclude that high ammonia levels at the blood-brain barrier reduce the efficiency of the glucose carrier in proportion to the length of exposure to moderately elevated blood ammonia concentrations, as seen in long-standing portal-systemic hyperammonaemia.

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D 32

Modulation of Pinocytosis by Sulfhydryl (SH) Compounds

By P. JOHANSSON and J.-O. JOSEFSSON. *Department of Pharmacology, University of Lund, Sweden*

The intensity of pinocytosis in *Amoeba proteus*, measured by the number of pinocytotic channels, is sensitive to several physical and chemical stimuli. Cation induced pinocytosis is inhibited by competing non-inducing cations (e.g. Ca) and by agents interfering with the biochemistry of mechanics of the pinocytotic process (e.g. inhibitors of metabolism and protein synthesis, Ca-binding agents and drugs which stabilize membranes or affect microtubules). By increasing the free intracellular Ca concentration (Ca_i^{++}) in the cell to a level above the optimum for channelformation some hormones and compounds (e.g. dbcAMP, the Ca-ionophore A 23187 and caffeine) inhibit pinocytosis in normal cells and activate pinocytosis in Ca-deficient cells. In contrast to these activating agents a few compounds (insulin, somatostatin, cGMP, morphine and metenkephalin) oppose blockade of pinocytosis caused by Ca_i (Johansson and Josefsson 1978 b).

Pretreatment of amoebae with 10⁻ M l-cysteine for 30 mins at pH 5 inhibits the cycle of pinocytosis induced by 100 mM MgCl at pH 6. The effect is potentiated by dbcAMP (10^{-4}M) or caffeine (10⁻ M) but reversed by addition of insulin (40 μE/ml, somatostatin (10^{-8} g/ml), cGMP (10^{-4} M) morphine (10^{-4} M) or met-enkephalin (10⁻ g/ml).

In the presence of cysteine intensive pinocytosis can be induced by Na in Ca-deficient cells only, while in normal cells channels will develop only when a calciumbinding agent is added to the inducer. Both verapamil (10 M) and procaine (or procainamide

suspended rat adipocytes glucagon is bound with an apparent K_d of 1–? nM and half time of dissociation of label is about 5 min (Sonne & Gliemann 1977) Glucagon in the suspension is degraded by proteases (unrelated to receptors) and the rate of degradation by this process is much higher than the total turnover of glucagon on the receptors (Sonne & Gliemann 1977) The following experiments were carried out in order to see whether a receptor mediated degradation existed in addition to the protease mediated ^{125}I-glucagon (0 6 nM) was incubated with adipocytes (?.5% (v/v) i e ?–3×10^5 cells/ml) for 30 min to achieve steady state of binding 0 ?% of the labelled glucagon in the suspension was bound to receptors and 0 0?% was bound to non saturable sites (i e in the presence of 1 μM unlabelled glucagon) Fig 1 left panel shows the elution profile on Sephadex G50-Fine of radioactivity extracted from cells incubated to steady state of binding. It appears that about 90% of the bound radioactive material elutes as iodoglucagon Fig 1 right panel shows the elution profile of radioactivity released from cells into non radioactive medium containing a large concentration of unlabelled glucagon which blocked protease mediated degradation of labelled glucagon It appears that about 60% elutes as glucagon whereas 40% elutes as small molecular weight components probably iodotyrosine

It is concluded that about 40% of the turnover of glucagon on receptors in rat adipocytes is accounted for by a process which involves binding degradation of the bound hormone and release of degraded fragments

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D 31

Ammonia Reduces Cerebral Extraction of Glucose in Advanced Portal-Systemic Hyperammonemia

By H LAURSEN N H DIEMER *and* A GJEDDE *Institutes of Neuropathology and Medical Physiology Dept A Copenhagen University Denmark*

ABnormally low brain-to-blood glucose concentrations ratios have been observed in certain forms of metabolic encephalopathy including rats with porta-caval anastomosis (PCA) of long duration A dysfunction of the transport mechanism that facilitates the diffusion of glucose from blood to brain was suggested in these cases and Fishman and Borton (1969) actually observed inhibition of the hexose membrane carrier in many organs including brain in ammonia intoxication Ammonia is generally believed to play a major role in the development of portal-systemic episodic encephalopathy and the sensitivity of the cerebral metabolism to a marked elevation of blood ammonia in PCA rats increases with length of exposure to moderately elevated blood ammonia levels (Gjedde *et al* 1978) Therefore the effect of ammonia on the transport of glucose from blood to brain was studied in rats with PCA for 4 or 8 weeks

We think that these specific increases in peroxisomal activity point to a role for peroxisomes during thermogenesis in brown fat. To our knowledge this is the first example of a physiologically stimulated increase in peroxisomal activity peroxisomal development (in liver) has hitherto only been stimulated pharmacologically by clofibrate treatment.

If this peroxisomal β-oxidation should play a role during thermogenesis the acetyl-CoA produced should be oxidised in the citric acid cycle in the mitochondria This means that the condensing partner oxaloacetate should be present in sufficient amounts to accept acetyl-CoA of both mitochondrial and peroxisomal origin The need for anaplerotic reactions is thus obvious.

We have therefore investigated the activity of pyruvate carboxylase in brown fat We found that this oxaloacetate-forming enzyme was present and had an activity of 40 nmol per min per mg mitochondrial protein and that it in isolated mitochondria could produce sufficient oxaloacetate not to limit the rate of fatty acid combustion. Further we could demonstrate by malonate inhibition of norepinephrine-stimulated respiration in brown adipocytes that its activity led to increased concentrations of citric acid cycle intermediates within the mitochondria.

We have found that pyruvate carboxylation is necessary for maximal thermogenesis to occur even when drainage of citric acid intermediates is eliminated. This means that the anaplerotic reaction is not used for synthetic reactions as is usually the case Rather the reaction observed is an *internal anaplerotic* one as defined by Scrutton (1975) A similar mechanism has been described for insect flight muscle (Crabtree *et al* 1972)

These results also explain observations on isolated brown adipocytes where the thermogenesis is stimulated by CO_2 bubbling (Pettersson 1977) and of an increased uptake of glucose into the brown fat *in vivo* during thermogenesis (Portet *et al* 1974) The glucose can be converted into pyruvate and together with bicarbonate be used for the internal anaplerotic reaction described.

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D 34

Extracellular K Concentration and Regional Blood Flow in Rat Brain Following 10 min of Cerebral Ischemia

By A J Hansen A. Gjedde and E Siemkowicz *Institute of Medical Physiology Dept A Panum Institute and Anesthe ia Dept H idovre Hospital Copenhagen Denmark*

The extracellular potassium concentration, K_e, i brain cortex increases during ischemia (Hansen 1978) but it is not known how K_e changes after an episode of brain ischemia.

10^{-3} M) which reduce the effects of dbcAMP and caffeine respectively diminish cysteine blockade of pinocytosis Cysteine on the other hand antagonizes the inhibition caused by colchicine (10^{-3} M) and activates the capacity for pinocytosis in starved cells and so imitates dbcAMP and caffeine Several other thiols and antioxidants viz. glutathione β-mercaptoethanol cysteamine l ascorbate (all 10^{-3} M) α tocopherol (2×10^{-4} M) and butylated hydroxytoluene (3×10^{-4} M) have effects similar to cysteine

Agents which oxidize or bind thiols e g diamide (10^{-} M) DTNB oxidized glutathione oxidized dithiothreitol sodium nitroprusside (all 10 M) and CdCl (10^{-4} M) antagonize the effects of cysteine Diamide inhibits pinocytosis in Ca -deficient cells and reverses caffeine blockade of pinocytosis These effects are antagonized by cysteine and ascorbate

The results indicate that SH groups are concerned with the central roles of Ca and cyclic nucleotides in pinocytosis Protection of SH groups or reduction of disulphides in the membrane and the cell interior may increase Ca_i and so activate and eventually inhibit channelformation Oxidation or reduction of critical SH groups within the tissue may therefore be a useful method for modulation of pinocytosis

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D 33

Thermogenesis in Brown Adipose Tissue The Possible Production of Acetyl-CoA in Peroxisomes and the Need for Internal Anaplerotic Reactions for Maximal Thermogenesis

By JAN NEDERGAARD STEFAN ALEXSON and BARBARA CANNON *The Wenner-Gren Institute University of Stockholm*

The substrate for heat production in brown adipose tissue is fatty acids When the tissue is stimulated by norepinephrine fatty acids are released from the triglyceride droplets within the tissue when these fatty acids are combusted heat is released

The breakdown of fatty acids to acetyl-CoA has been thought to occur exclusively through mitochondrial β-oxidation Recently however Lazarow and de Duve (1976) found β-oxidation occurring in the peroxisomes in rat liver We have examined whether peroxisomal β-oxidation could occur in brown adipose tissue

We found that the peroxisomal cyanide insensitive NAD-dependent palmitoyl-CoA oxidase was present in brown fat *We have followed this enzyme* as well as the classical peroxisomal enzyme catalase and the mitochondrial enzyme cytochrome oxidase during cold-adaptation of rats and compared the activity of these enzymes in cold-adapted (3 weeks at 5°C) and control (3 weeks at 22°C) rats Whereas cytochrome oxidase during cold-adaptation increased to 160% of the controls (per g wet weight) palmitoyl-CoA oxidase increased to 230% and catalase to 460% If the values are *related to the total* amount of brown fat in the rat the increases were to 380% 610% and 950% respectively

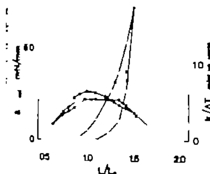


Fig. 1 ○ Relaxed wall tension, T_{rel} (Vessel held in Ca-free solution). ● Active wall tension, $\Delta T = T_{act} - T_{rel}$, where T_{act} is activated wall tension (vessel held in K-rich solution containing 40 μM noradrenaline) ▲ Ratio of active dynamic stiffness, k, (difference between dynamic stiffness when activated and when relaxed) to ΔT. Values plotted against relative internal circumference L/L_0. Vessel was activated for 2 min every 15 min and internal circumference set to given values in the order indicated by arrows while vessel was relaxed. ■ ◆ ΔT and k/ΔT respectively measured in two activations at end of experiment. $L_0/\pi = 160$ μm. Segment length = 0.73 mm. Active stiffness at L_0 was $40\ \Delta T_0/L_0$. Temperature 37°C.

$L/L_0 = 1.64 \pm 0.11$ (N=5). Here k was proportional to ΔT. Below L_0, ΔT fell with decreasing L, but the ratio k/ΔT increased.

The results suggest that the fall in ΔT above L_0 could indeed be due to a reduction in overlap of contractile filaments, but that below L_0 the fall may in part be due to steric interference between such filaments.

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D 36

A Local Desaturation, Residue Detection Method for Simultaneous Measurements of Regional Hepatic Blood Flow and Distribution Volumes for Extracellular Indicators

By WILLIAM P. PAASKE. *Institute of Medical Physiology B. University of Copenhagen, Denmark*

The present study was undertaken in order to determine blood flow and distribution volumes for extracellular hydrophilic indicators in minute areas of the liver.

The experiments were performed in rabbits anaesthetized with sodium pentobarbitone. A median abdominal incision was made in order to expose the liver. The hepatic artery and vein were clamped. 50 μl isotonic saline containing about 0.5 mCi ^{51}Cr-ethylene-diamine-tetraacetate (^{51}Cr EDTA) was applicated to the surface of the liver for 5 min during which time the exposed areas were covered with polyethylene sheets. After this period the sheets were removed and the area was rinsed with isotonic saline. 50 μl isotonic saline containing about mCi ^{133}Xenon was then applicated to the field which was immediately covered with

Ischemia was induced in halothane anestetized normoglycemic rats by inflation of a pneumatic cuff placed around the neck and lowering of blood pressure by bleeding (Siemkowicz and Hansen 1978) After craniotomy of the left parietal bone K_e was measured in the cortex and basal ganglia by means of K -selective microelectrodes Cerebral blood flow was determined by the method of Schaefer *et al* (1976) A bolus of ^{14}C-butanol was rapidly injected in a femoral vein and arterial blood was sampled continuously for 20 s until heart action was quickly arrested by i v injection of saturated KCl ^{14}C activity in the brain was measured for calculation of regional blood flow

During ischemia K_e increased identically in the two regions An initial slow increase to 10 mM for 2 min was followed by a rapid increase to 70 mM and a final slow increase to 80 mM

After 10 min the cuff was deflated and blood reinfused Adrenaline was given when nessesary in order to maintain blood pressure above 100 mmHg K_e reached preischemic levels after 4 min in the cortex and after 2 min in the basal ganglia The decrease of K_e displayed two phases A slow phase from 80 to 50 mM for 3 min in the cortex and for 1 min in the basal ganglia, and a second rapid decline from 50 to 3 mM lasting 1 min in bot regions After about 1 min the blood flow was 21 ml/100 g/min in the cortex and 155 ml/10 g/min in the basal ganglia but increased to similar high levels in the cortex when the K was normalized

The experiments thus suggest an intimate relationship between cerebral blood flow an the extracellular potassium concentration after ischemia

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D 35

Mechanical Evidence for a Sliding Filament Mechanism in Vascular Smooth Muscle

By M J MULVANY and D M WARSHAW *Biophysics Institute Aarhus University Denmark*

The structural evidence (Ashton et al 1975) for a sliding filament mechanism in smooth muscle has been tested by determining the effects of stretch on active isometric wall tension ΔT and active dynamic stiffness k Rat mesenteric arterial resistance vessel segments were mounted on a myograph (Mulvany & Halpern 1976) In 5 experiments similar to that shown in Fig. 1 ΔT and k were measured in the range 0 6 L_o to 1 6 L_o where L_o is the internal circumference L, giving peak active isometric wall tension ΔT_o. Over this range the changes determined were essentially reversible Above L_o ΔT fell almost linearly with increasing L, the points lying close to a line which cut the abscissa at

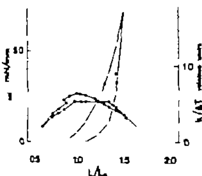


Fig. 1 ○ Relaxed wall tension, T_{rel} (Vessel held in Ca-free solution). ● Active wall tension, $\Delta T = T_{act} - T_{rel}$, where T_{act} is activated wall tension (vessel held in K-rich solution containing 40 μM noradrenaline). ▲ Ratio of active dynamic stiffness, k, (difference between dynamic stiffness when activated and when relaxed) to ΔT. Values plotted against relative internal circumference L/L_0. Vessel was activated for 2 min every 15 min and internal circumference set to given values in the order indicated by arrows while vessel was relaxed. ■ ◆ ΔT and k/ΔT respectively measured in two activations at end of experiment. $L_0/\pi = 160$ μm. Segment length = 0.73 mm. Active stiffness at L_0 was $40\ \Delta T_0/L_0$. Temperature 37°C

$L/L_0 = 1.64 \pm 0.11$ (N = 5). Here k was proportional to ΔT. Below L_0, ΔT fell with decreasing L, but the ratio k/ΔT increased.

The results suggest that the fall in ΔT above L_0 could indeed be due to a reduction in overlap of contractile filaments, but that below L_0 the fall may in part be due to steric interference between such filaments.

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D 36

A Local Denaturation, Residue Detection Method for Simultaneous Measurements of Regional Hepatic Blood Flow and Distribution Volumes for Extracellular Indicators

By WILLIAM P. PAASKE. *Institute of Medical Physiology B, University of Copenhagen, Denmark.*

The present study was undertaken in order to determine blood flow and distribution volumes for extracellular hydrophilic indicators in minute areas of the liver.

The experiments were performed in rabbits anaesthetized with sodium pentobarbitone. A median abdominal incision was made in order to expose the liver. The hepatic artery and vein were clamped. 50 μl isotonic saline containing about 0.5 mCi ^{51}Cr-ethylene-diamine-tetraacetate ^{51}Cr-EDTA, was applicated to the surface of the liver for 5 min during which time the exposed areas were covered with polyethylene sheets. After this period the sheets were removed and the area was rinsed with isotonic saline. 50 μl isotonic saline containing about mCi ^{133}Xenon was then applicated to the field which was immediately covered with

Ischemia was induced in halothane anestetized normoglycemic rats by inflation of a pneumatic cuff placed around the neck and lowering of blood pressure by bleeding (Siemkowicz and Hansen 1978) After craniotomy of the left parietal bone K_e was measured in the cortex and basal ganglia by means of K^+ selective microelectrodes Cerebral blood flow was determined by the method of Schaefer *et al* (1976) A bolus of ^{14}C-butanol was rapidly injected in a femoral vein and arterial blood was sampled continuously for 20 s until heart action was quickly arrested by i v injection of saturated KCl ^{14}C activity in the brain was measured for calculation of regional blood flow

During ischemia K_e increased identically in the two regions An initial slow increase to 10 mM for 2 min was followed by a rapid increase to 70 mM and a final slow increase to 80 mM

After 10 min the cuff was deflated and blood reinfused Adrenaline was given when nessesary in order to maintain blood pressure above 100 mmHg K_e reached preischemic levels after 4 min in the cortex and after 2 min in the basal ganglia. The decrease of K_e displayed two phases A slow phase from 80 to 50 mM for 3 min in the cortex and for 1 min in the basal ganglia, and a second rapid decline from 50 to 3 mM lasting 1 min in both regions After about 1 min the blood flow was 21 ml/100 g/min in the cortex and 155 ml/100 g/min in the basal ganglia but increased to similar high levels in the cortex when the K_e was normalized

The experiments thus suggest an intimate relationship between cerebral blood flow and the extracellular potassium concentration after ischemia

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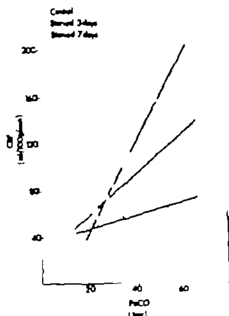
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D 35

Mechanical Evidence for a Sliding Filament Mechanism in Vascular Smooth Muscle

By M J MULVANY and D M WARSHAW *Biophysics Institute Aarhus University Denmark*

The structural evidence (Ashton et al 1975) for a sliding filament mechanism in smooth muscle has been tested by determining the effects of stretch on active isometric wall tension ΔT and active dynamic stiffness k Rat mesenteric arterial resistance vessel segments were mounted on a myograph (Mulvany & Halpern 1976) In 5 experiments similar to that shown in Fig. 1 ΔT and k were measured in the range 0 6 L_o to 1 6 L_o where L_o is the internal circumference L giving peak active isometric wall tension ΔT_o. Over this range the changes determined were essentially reversible Above L_o ΔT fell almost linearly with increasing L, the points lying close to a line which cut the abscissa at



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D 38

Decreased Sympathetic Vasomotor Tone in Diabetic Orthostatic Hypertension

By J. HILSTED. *Institute of Medical Physiology B. University of Copenhagen and Hvidøre Hospital. Denmark.*

In normals, subcutaneous blood-flow in the ankle region, measured by means of the ^{133}Xe washout technique, decreases 60% when the position of the ankle is changed from cardiac level to 50 cm below the heart. A sympathetic axon reflex is responsible for this flowreduction.

The aim was to determine if this reflex control of blood-flow is intact in long-term juvenile diabetics.

A normal response (blood-flow reduction 53 (36–58) % (mean and range)) was found in 6 diabetics without neuropathy, age 25 years (mean), duration of diabetes 12 years (mean). In 9 diabetics with autonomic neuropathy (beat-to-beat variation in heart rate < 10/min during

a gas tight mylar membrane and polyethylene sheets The clamp was removed giving a period of occlusion which had a total duration of about 5.5 min The washouts of ^{51}Cr EDTA and ^{133}Xenon were recorded by residue detection using a one inch NaI(Tl) scintillation detector After cross-talk and background corrections the initial slopes of the washout curves were determined by the "least squares" method The blood flow was calculated from the equation $f(bl) = k_{Xe} \cdot \lambda_{Xe} \cdot 100$ (ml/100 g min) where f(bl) is blood flow k_{Xe} is the rate constant of the initial slope (min^{-1}) and λ is the blood to tissue partition coefficient of ^{133}Xenon (0 7 ml/g) The distribution volume of ^{51}Cr EDTA V_d was calculated from the equation $V_d = f(pl) \cdot \bar{t}$ where f(bl) is plasma flow and $\bar{t}$ is the mean transit time of Cr EDTA $\bar{t}$ was taken as the reciprocal value of the rate constant of the initial slope of the ^{51}Cr EDTA washout Washout of ^{51}Cr EDTA was considered to be blood flow limited

Blood flow was 93 4 ml/100 g min (S E 23 6 n=9) At a hematocrit value of 0 370 (S E 0 010) this corresponds to a plasma flow of 53 9 ml/100 g min (S E. 15 7) V_d was 23 0 ml/100 g (S E 1 5) which is in agreement with the value found by Goresky (1963) in the dog by an independent multiple indicator dilution method using sucrose as indicator

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D 37

Reduced Cerebral Blood Flow Response to Carbon Dioxide in Fasting Rat

By A GJEDDE and S M DE LA MONTE *Institute of Medical Physiology Dept A Copenhagen University Denmark*

The contribution of central and peripheral noradrenergic neurons to the regulation of cerebral blood flow is the subject of dispute (Heistad and Marcus 1978) Reserpine treatment of rats established no role for these neurons in the control of normal cerebral circulation although the response to hypocapnia was reduced during hypotension (Caronna *et al* 1975)

Starvation in rat causes suppression of the sympathetic nervous system (Young and Landsberg 1977) Therefore we studied the response of the cerebral circulation to acute changes of arterial carbon dioxide tension (Pa_{CO_2}) in rats starved for 3 or 7 days Whole brain blood flow (CBF) was measured by withdrawal of arterial and cerebral venous blood during *washout of xenon in* rats anesthetized with halothane and ventilated with varying amounts of CO_2 (Gjedde *et al* 1975) The result is shown in Figure 1 The $\Delta CBF/\Delta Pa_{CO_2}$ ratio declined from 4% in control rats to 1 6% in rats starved for 3 days and was virtually abolished (0 6%) in rats starved for 7 days

We conclude that the sympathetic nervous system affects the cerebral circulation under abnormal conditions such as *hypotension or starvation*

but the capillary density of the soleus muscle is less than in other extremity muscles (Andersen and Kroese 1978) The tortuous arrangement of the capillaries in the soleus muscle can give this muscle a relatively larger capillary volume On the other hand the large size muscle fibres of the soleus muscle will result in an on a relative basis smaller surface area. It is then concluded that the difference that exist in extra-cellular water volume between the two muscle studied can only partly be explained by the differences in fibre composition, and factors such as fibre size and capillarization (number and length) which varies between muscles of man may be equally important.

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hyperventilation) age 32 years (mean) duration of diabetes 20 years (mean) the reduction in blood-flow upon lowering of the ankle was slightly diminished (blood flow reduction 42 (26–93)%) A marked deterioration of the response was found in 6 diabetics with autonomic neuropathy including orthostatic hypotension in whom blood-flow *increased* to 108 (65–164)% age 39 years (mean) duration of diabetes 23 years (mean) Ankle blood-flow at cardiac level was similar in the three groups

In conclusion in patients with diabetic orthostatic hypotension the subcutaneous axon reflex is absent *which may be one of several factors responsible for their orthostatic* symptoms

D 39

Inulin Space in Red and White Skeletal Muscle of Man

By GISELA SJOGAARD and BENGT SALTIN *August Krogh Institute University of Copenhagen Denmark*

In species like the rat two main skeletal muscle fibres types red and white can be found with distinct differences in their metabolic profiles Further differences in intra- and extra-cellular water and electrolyte distribution have been demonstrated (Sréter and Woo 1963)

Also in man two main fibre types can be found the soleus muscle being predominantly composed of "red" fibres while the triceps brachii muscle is predominantly white These muscles were sampled from 6 healthy young males and 6 healthy young females Muscle fibre type composition was based on the staining for myofibrillar ATPase "red = slow (ST) and "white = fast (FT) The shortest diameter was used for calculation of fibre area. The total water was obtained by weighing the muscle sample before and after drying Extra-cellular volume (H_2O_E) was measured as inulin distribution space in the supine position using an intravenous dose of 1 mCi tritiated inulin and with blood and muscle sampling after 1 hour (Sjøgaard submitted)

All subjects had higher % ST fibres in the soleus than in the triceps muscle mean values (range) being 73 (51–90)% St and 39 (19–60)% ST respectively ($p<0\ 05$) Mean fibre diameter was larger in the soleus muscle giving approximately 50% larger fibre areas in the soleus as compared to the triceps muscle Total muscle water averaged 3?1 (?99–337) and 318 (295–333) ml/100 g dry weight for the soleus and triceps muscles respectively ($p>0\ 05$) The extra-cellular space however was larger in the soleus than in the triceps muscle mean values (range) 55 (38–7?) and 42 (18–62) ml/100 g dry weight respectively ($p<0\ 05$) None of the variables were significantly different between males and females

There was a relationship between % ST fibres and $H\ O_E$ but it was equally evident that the data points for the two muscles were grouped separately which means that at the same per cent ST fibre H_2O_E was lowest in the triceps muscle

As an explanation for the present finding factors like capillarization and fibre sizes of the two muscles may contribute There are slightly more capillaries around ST than FT fibres